# Transglutaminase expression and activity in MC3T3-E1 osteoblast cultures

**Céline Lefebvre**

A thesis submitted to the faculty of Graduate Studies and Research
In partial fulfillement of the requirements for the degree

of

Master of Science

© Céline Lefebvre, June 2004

Department of Anatomy and Cell Biology
McGill University
Montreal, Quebec, Canada

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

*Your file Votre référence*
*ISBN: 0-494-06416-1*
*Our file Notre référence*
*ISBN: 0-494-06416-1*

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

---

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA: | ascorbic acid |
| ALP: | alkaline phosphatase |
| α-MEM: | alpha minimum essential medium |
| AP: | activating peptide |
| BAG-75: | bone acidic glycoprotein-75 |
| BCA: | bicinchonic acid |
| βGP: | beta-glycerolphosphate |
| bPA: | biotinylated primary amine |
| BSA: | bovine serum albumine |
| BSP: | bone sialoprotein |
| COLLI: | collagen type I |
| DNA: | deoxyribonucleic acid |
| DTT: | dithiothreitol |
| ECL: | enhanced chemiluminescence |
| EDTA: | ethylenediaminetetraacetic acid |
| ECM: | extracellular matrix |
| FBS: | fetal bovine serum |
| FXIIIa: | factor XIIIa |
| FN: | fibronectin |
| GAPDH: | glyceraldehyde 3-phosphate dehydrogenase |
| GTP: | guanosine triphosphate |
| HA: | hydroxyapatite |
| HCl: | hydrochloric acid |
| HRP: | horseradish peroxidase |
| IGEPAL: | polyoxyethylene nonyl phenol |
| ISOP: | isopeptide bond |
| LRW: | London resin white |
| NaCl: | sodium chloride |
| NCP: | noncollageneous proteins |
| OCN: | osteocalcin |
| OD: | optical density |
| ON: | osteonectin |
| OPN: | osteopontin |
| PBS: | phosphate-buffered saline |
| PCA: | perchloric acid |
| PLP: | periodate/lysine/paraformaldehyde |
| PMSF: | phenylmethylsulfonyl fluoride |
| pNPP: | ρ-nitrophenylphosphate |
| PPC: | polypeptide chain |
| PVDF: | polyvinylidene difluoride |
| RNA: | ribonucleic acid |
| RPM: | revolutions per minute |
| RT-PCR: | reverse transcription polymerase chain reaction |
| SDS-PAGE: | sodium dodecyl sulphate polyacrylamide gel |

| | |
|---|---|
| TCA: | trichloroacetic acid |
| TBS: | Tris-buffered saline |
| TBS-T: | Tris-buffered saline-Tween |
| TH: | thrombin |
| TGase: | transglutaminase |
| TG1: | transglutaminase type 1 |
| TG3: | epidermal-type transglutaminase |
| TG5: | transglutaminase type 5 |
| tTG: | tissue transglutaminase |
| Tween 20: | polyoxyethylene 20-sorbitan monolaurate |
| XRD: | X-ray diffraction |

# ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors, Dr. Marc McKee and Dr. Mari Kaartinen, for providing me the opportunity to work in their laboratory, and for their guidance and support over the course of my studies. Thanks to both of my supervisors for providing me an enjoyable learning environment as well as invaluable tools and experiences. I am grateful to the members of the laboratory, both past and present, for their friendship, support, and for extremely precious daily discussion and criticism. I would like to give a special thanks to my very good friend and colleague, Ms. Jennifer Forsprecher, for her enjoyable presence in the lab and for the happiness she brought to my life.

I want to acknowledge Dr. Renny Franceschi (University of Michigan, Ann Harbor, MI, USA) who generously provided the cell line used in this study, and also for taking the time to have me visit his laboratory. Special thanks to Dr. Nathalie Lamarche and the members of her laboratory, for providing me with work space, work equipment, helpful discussions, and to making the time spent in and out of the lab more enjoyable. Thanks to Dr. Louise Larose and Dr. Michael Greenwood for useful advice on experimental design and techniques.

Finally, I would like to thank my parents, Yves and France, whose love and support helped me reach my goals. The completion of this work could not have been possible without their encouragement and understanding.

# PREFACE (contribution of authors)

The research presented in this thesis is entirely my own with the following exception:

The X-Ray diffraction analysis presented in Figure 15 and the description of the method in section 3.10 were kindly done by fellowstudent Yung-Chin Chien.

# ABSTRACT

Transglutaminases are enzymes that stabilize extracellular matrices by catalyzing the formation of protease-resistant isopeptide crosslinks between and within their substrate proteins. Two transglutaminase isoforms, tissue transglutaminase (tTG) and factor XIIIa (FXIIIa), are expressed in skeletal tissues. Tissue transglutaminase has been previously localized in bone to osteoblasts, the osteoid layer and the pericellular matrix of osteocytes. Factor XIIIa has been localized to mineralizing chondrocytes, however, its expression in osteoblasts has not been reported yet. In order to understand the role of transglutaminases in hard tissue formation, we have investigated tTG and FXIIIa expression and activity during osteoblast differentiation and mineralization using the well-characterized MC3T3-E1 preosteoblast cell line. We show that tTG and FXIIIa are expressed at all time points examined in the cell cultures, and that their expression and activity does not appear to be significantly changed by the differentiation or mineralization state of the cells. We show that tTG localizes homogeneously throughout the cells and the extracellular matrix, whereas FXIIIa distribution is polarized to one side of the cells at earlier time points, but becomes incorporated into the matrix produced by differentiating cells in mature cultures. These expression profiles suggest a dual role, initially in cell adhesion and attachment and, later, in protein assembly, where the transglutaminases likely stabilize the matrix and assist in the formation of an extracellular matrix competent for subsequent mineralization. The identification and characterization of two transglutaminase isoforms in osteoblasts suggest a potentially overlapping roles for these two enzymes in the MC3T3-E1 cell culture system.

# RÉSUMÉ

Les transglutaminases sont une famille d'enzymes qui contribuent a la stabilisation des matrices extracellulaires en catalysant la formation de liens peptidiques entre leurs substrats. Les polymères de protéines ainsi formés sont très stables et résistants aux protéases. Deux isoformes, la "tissue transglutaminase" et la "transglutaminase facteur XIIIa", sont exprimées dans les tissus squelettiques. Des recherches précédentes ont démontré que, dans le tissu osseux, la tissue transglutaminase est localisée dans les ostéoblastes, l'espace péricellulaire dans ostéocytes et dans l'ostéoïde. Le facteur XIIIa a été localisé dans le cartilage en minéralisation, toutefois, son expression n'a jamais été identifiée dans les ostéoblastes. Afin de mieux comprendre le role des transglutaminases dans la formation des tissus calcifiés, nous avons investigué l'expression et l'activité des deux isoformes (tissue transglutaminase et facteur XIIIa) lors du processus de différentiation et de minéralisation des ostéoblastes. Pour ce, nous avons utilisé la lignée cellulaire de pré-osteoblastes MC3T3-E1. Dans cette étude, nous montrons que les deux isoformes de transglutaminase sont exprimées et sont actives en tout temps dans le système de culture cellulaire, peu importe le stade de différentiation ou de minéralisation des cellules. La tissue transglutaminase est localisée uniformément dans la matrice et les cellules, tandis que le facteur XIIIa a une distribution plus spécifique. Dans les jeunes cultures, sa localisation est polarisée à un côté des cellules, alors que dans les cultures matures d'ostéoblastes différenciés, le facteur XIIIa s'incorpore dans la matrice. Ces résultats suggèrent que, dans les jeunes cultures d'ostéoblastes, la transglutaminase est impliquée dans le processus d'adhésion cellulaire. Dans les ostéoblastes matures, la transglutaminase a probablement un rôle dans la stabilisation de la matrice extracellulaire, où elle participerait à l'assemblage adéquat des protéines pour préparer la matrice pour à la minéralisation. Aussi, le fait que nous démontrons l'expression des deux isoformes de transglutaminase dans les ostéoblastes suggère une redondance de leurs fonctions.

# 1. INTRODUCTION

## *1.1 Bone formation and bone cell overview*

Bone is a specialized form of connective tissue composed of cells and an abundant extracellular matrix (ECM). The ECM has the ability to calcifiy, resulting in the typical hardness of bone. Bone is formed by two different processes, endochondral and intramembranous ossification. Most of the long and short bones (e.g.: tibia, femur) are formed by endochondral ossification where the initial condensation of mesenchymal cells initially produces a cartilage model. The cartilagenous template calcifies and is subsequently replaced by a highly organized and compact bone produced by osteoblasts. Flat bones (e.g.: skull, mandible) are formed by intramembranous bone formation where mesenchymal cells directly differentiate into osteoblasts.

After the skeleton has reached its full mass and volume, bone mass and quality is maintained by a continuous process of bone remodeling, involving cycles of bone resorption and bone formation. Bone remodeling is controlled by bone cells, namely bone-lining cells, osteoblasts, osteocytes and osteoclasts under the control of various hormones, cytokines and growth factors. Bone-lining cells are relatively inactive, flattened cells that reside at the bone surface. Osteoblasts form osteoid, an initially uncalcified bone matrix, and become trapped in their lacunae upon its mineralization and are thereafter known as osteocytes.

Bone resorption is achieved by osteoclasts, which are multinucleated cells formed from hematopoietic precursors. On the bone surface and after fusion of monocytes, osteoclasts become polarized and form a sealing zone and ruffled borders under which bone resorption takes place. Osteoclasts create a sealed acidic environment that, together with the action of low pH and secreted enzymes, demineralizes the inorganic phase and degrades bone matrix. Following osteoclastic bone resorption, osteoblasts are subsequently recruited to the resorbed site to form new bone.

## 1.2 Bone matrix proteins

Bone ECM is composed of an inorganic mineral phase and an organic phase. The mineral phase consists of carbonate-substituted hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) deposited within the organic matrix, which is mainly (80-90%) collagen. The most abundant collagen types are type I (95%) and type V (5%). The organic phase of bone also contains 5-10% noncollageneous proteins (NCPs) and proteoglycans (such as decorin and biglycan), which are thought to contribute to the integrity of the tissue and to regulate bone remodeling and mineralization[1,2]. These NCPs are acidic proteins and phosphoproteins, such as osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP), matrix Gla protein, osteonectin, and bone acidic glycoprotein-75 (BAG-75). Bone cells are continuously interacting with this inorganic-organic environment and respond to signals coming from cell-cell, and cell-matrix interactions. However, it is not fully known how cellular activity is coordinated to secrete and assemble the proteins required to form an ECM that accomodates mineral deposition and growth.

Some key NCPs have been identified as *in vitro* regulators of mineral homeostasis. A very abundant NCP, BSP, is one of such proposed regulatory protein. BSP contains an Arg-Gly-Asp (RGD) cell-binding motif that allows the protein to bind integrin receptors on the cell surface and thereby might mediate cell attachment and cell-cell or cell-matrix communication[3]. Its pattern of expression is essentially restricted to bone, teeth, and hypertrophic cartilage[4]. In the mineralized bone matrix, BSP is more abundant at sites where new bone is formed[5]. BSP has been shown *in vitro* to bind hydroxyapatite with high affinity and to function as a nucleator of hydroxyapatite crystals[6]. Another major phosphoprotein in the ECM of bone is OPN. Like BSP, OPN has an RGD sequence that suggests a role in cell adhesion and mediation of signals through an interaction with cell surface integrins[7]. OPN is expressed in a variety of tissues, including bone, teeth, kidney, brain, and the vasculature[8]. OPN is distinguished from other NCPs by its unique distribution in mineralized tissues, where it preferentially accumulates with nascent mineral crystals and at bone surfaces and matrix-matrix, matrix-mineral and matrix-cell interfaces[9]. These observations suggest a role for OPN in the regulation of bone mineralization in the bulk ECM, and in terminating mineralization

at bone surfaces. These suggested roles are supported by the work of Hunter and colleagues (1993)[6] who demonstrated that OPN is an effective inhibitor of hydroxyapatite formation and growth *in vitro*. Of particular relevance to the work reported here, OPN and BSP are substrates for tranglutaminase enzymes.

## 1.3 Transglutaminases

Transglutaminases (TGases) (EC 2.3.2.13) are a family of enzymes that catalyze the $Ca^{2+}$- dependant formation of intramolecular and intermolecular isopeptide bonds between the γ-carboxamide of peptide-bound glutamine and the ε-amino group of lysine, or a primary amine such as putrescine, spermine, and spermidine. The resulting ε-(γ-glutamyl)lysine crosslink is highly resistant to proteolytic degradation and mechanical stretch (Figure 1). Crosslinking between proteins results in the formation of high molecular weight protein polymers. To date, nine different TGase isoforms have been reported in mammals[10]. TGases share common structural and functional features such as a lack of glycosylation and disulphide bonds, and have homologous active sites. The overall tertiary structure of some of the isoforms reveals a monomer with four distinct domains, which is thought to be conserved among all TGases[11]. The active site of the enzymes involve a catalytical triad of Cys-His-Asp or Cys-His-Asn, where the cysteine residue (Cys) and its thiol group are critical for the crosslinking reaction. The presence of calcium ions is also necessary for the transamidation activity. Upon calcium binding, TGases undergo a conformational change that unmasks the reactive cysteine residue at the active site. Despite their similar catalytic mechanism, TGases family members differ in their substrate specificity. However, the search for a consensus sequence around glutamine and lysine substrate sites for TGases has remain unsuccessful[12]. It has been suggested that the ability of some proteins to act as TGase substrates depends on the chemical nature of the side chain residues surrounding the substrate residues, or the charges of the protein at the tertiary structure level. It is also possible that proteolytic cleavage or other post-translational modifications of substrate proteins affect the availability of certain lysine and glutamine residues for the crosslinking reaction.

The TGase isoforms have a specific tissue distribution, but each isoform can be expressed in many tissues, often in combination with other TGases. Because of the specificity of tissue distribution and the availability of protein substrates for the nine TGase isoforms, various important physiological functions can result from a common crosslinking reaction. Functions include blood coagulation, stabilization of extracellular matrices, cell-matrix interaction, terminal differentiation of keratinocytes, amongst others, as summarized in Table 1. To date, however, most of the TGase isoforms have unknown or only partially characterized functions.

**Table 1. Transglutaminase isoforms characteristics.** Adapted from Griffin et al (2002)[22] and Lorand et al (2003)[10].

| Protein | Synonyms | Localization | Tissue expression | Prevalent Function |
|---|---|---|---|---|
| Factor XIIIa | Fibrin-stabilizing factor, Blood coagulation factor XIII subunit A | Cytosolic, extracellular | Platelets, astrocytes, chondrocytes, placenta, plasma, synovial fluid | Blood coagulation, wound healing, bone growth |
| TG1 | Keratinocyte TG | Membrane, cytosolic | Keratinocytes, brain | Cell-envelope formation |
| TG2 | Tissue TG | Cytosolic, nuclear, membrane, cell surface, extracellular | Ubiquitous | Multiple |
| TG3 | Epidermal TG | Cytosolic | Squamous epithelium, brain | Cell-envelope formartion |
| TG4 | Prostate TG | Unknown | Prostate | Semen coagulation in rodents |
| TG5 | $TG_X$ | Unknown | Ubiquitous except for CNS and lymphatic system | Epidermal differentiation |
| TG6 | $TG_Y$ | Unknown | Unknown | Unknown |
| TG7 | $TG_Z$ | Unknown | Ubiquitous | Unknown |
| Band 4.2 | Erythrocyte membrane protein band 4.2 | Membrane | Red blood cells, bone marrow, fetal liver, spleen | Membrane cytoskeletal component |

## 1.4 Tissue transglutaminase

Tissue transglutaminase (tTG, TG2, TGc) was the first TGase to be discovered and it has been extensively studied over many years. It is ubiquitously expressed in mammalian tissues[13] and it has a widespread protein distribution. It is a monomeric enzyme found predominantly in the cytoplasm of cells, but it has also been observed at the plasma and nuclear membrane. Even though tTG lacks the hydrophobic leader sequence characteristic of secreted proteins, it has been localized extracellularly at the cell surface and in the ECM[14]. It is possible that a posttranslational modification involving a N-terminus acetylation of the enzyme serves as signal sequence for its secretion[15]. tTG is constituvely expressed in many cell types[16] and it has a high basal promoter activity[17]. However, its expression level does not always correlate with enzymatic activity. For example, Piacentini et al (1992)[18] have demonstrated that overexpression of tTG does not lead to increased amounts of crosslinked products. This may be explained by the multiplicity of factors influencing tTG expression and activity. Tissue transglutaminase expression is mainly adjusted by changes at the transcriptional level. The promoter contains responsive elements for various inducers and inhibitors, including retinoic acid, dimethyl sulfoxide and transforming growth factor-ß. The methylation status of the tTG promoter may also influence tTG expression. At another level, tTG activity is known to be modulated by guanosine nucleotides (GTP) (inhibitor) and by $Ca^{2+}$ (activator).

The complexity of tTG regulation reflects the diversity of its biological roles. Its crosslinking dependent functions are described in general as ECM stabilization, cell adhesion, wound healing, formation of the dermo-epidermal junction, development of skeletal elements, and apoptosis[19]. Tissue transglutaminase is distinguished from other TGases by its ability to bind and hydrolyze GTP in a manner similar to G proteins[13]. Recently, tTG has been reported to function as a protein disulphide isomerase, introducing disulphide bridges in newly translated proteins to allow proper conformation[20]. As the distribution and the proposed functions of tTG were so diverse and seemingly important, it was thought that the knock-out of tTG alleles would be lethal.

Surprisingly, the targeted inactivation of tTG in mice reveals the absence of a distinct developmental phenotype[21]. These findings allude to an important concept shown for other proteins: that there is a possible redundancy of function amongst the TGase isoforms in various tissues. Although tTG has been studied extensively, its precise physiological role remains unclear.

One approach to better understanding the role of tTG is to characterize and localize its substrate proteins (Table 2). For example, the identification of fibronectin (FN) as a tTG substrate has led to several hypotheses, suggesting an important role for tTG in cell adhesion[22]. Griffin et al (2002)[22] have extensively addressed this possibility. They have shown that endogenous tTG crosslinks cellular FN, converting soluble FN to insoluble, polymerized fibronectin fibrils[23] shown to facilitate cell adhesion, thus implicating tTG in this process. This hypothesis has been supported by the observation that tTG colocalizes with β1 integrin during cell spreading events[24]. The β1 integrin is known to be implicated in cell adhesion and FN assembly. Akimov et al (2000, 2001)[25,26] clarified the role of tTG in cell adhesion when they found that tTG independently acts as a coreceptor for the association of integrins with fibronectin due to its high affinity for both proteins. In fact, the polymerization of FN in cell adhesion is a tTG-independent process, which is initiated by the binding of FN to integrins. This function does not require a crosslinking reaction, suggesting that tTG function in cells is not strictly restricted to its known crosslinking activity.

Similarly, the identification of substrate proteins for tTG in bone and cartilage suggest a role for tTG in matrix stabilization and regulation of matrix mineralization. Osteonectin (ON) is a predominant substrate for tTG in cartilage[36], and OPN, a protein thought to be involved in the regulation of matrix mineralization, is also a substrate for tTG[27]. Moreover, new tTG substrates have been identified in bone, namely BSP, a mineral nucleator, and $\alpha_2$HS-glycoprotein[28], a mineral inhibitor in soft tissues that accumulates in bone due to its affinity for mineral.

**Table 2. Noncollagenous substrate proteins for tissue transglutaminase and factor XIII in cartilage and bone.** tTG: tissue transglutaminase; FXIII: factor XIII. Adapted from Aeschlimann et al (1996)[29].

| Noncollagenous protein | Enzyme | Reference |
|---|---|---|
| Bone sialoprotein | tTG | Kaartinen et al (2002)[28] |
| $\alpha_2$ HS-glycoprotein | tTG | Kaartinen et al (2002)[28] |
| Fibrillin | tTG | Aeschlimann et al (1996)[29] |
| Fibronectin | tTG and FXIIIa | Verderio et al (1998)[23] |
| Osteonectin | tTG | Aeschlimann et al (1995)[42] |
| Osteopontin | tTG and FXIIIa | Prince et al (1991)[27] |

## *1.5 Factor XIIIa*

Factor XIII (FXIII), also refered to as the blood coagulation factor, is the second most widely distributed member of the TGase family. Unlike tTG, FXIII apparently has one well-defined physiological function. FXIII is found in plasma as an inactive, tetrameric molecule consisting of two «A» catalytic subunits and two «B» non-catalytic subunits. FXIII is also found in megakaryocytes, bone marrow cells, monocytes, and monocytes-derived macrophages. Its cellular form is a homodimer of two «A» catalytic subunits (FXIIIa). Its precise site of synthesis is however controversal[30]. FXIIIa is activated by thrombin cleavage at the Arg-37-Gly-38 site, followed by the release of a 4 kDa activating peptide (AP). The modified «A» subunits represent the active configuration (see Figure 2). Thrombin-activated FXIIIa crosslinks fibrin during the last step of the blood coagulation cascade, forming a highly resistant blood clot[31]. FXIIIa can also be activated in a non-proteolytic manner. In the presence of physiological concentrations of $Ca^{2+}$, cellular FXIIIa undergos a slow and progressive activation[32]. Other divalent metal ions such as $Zn^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ have been reported to substitute

for $Ca^{2+}$ at different levels of efficiency[33]. A third way of activating intracellular FXIIIa has been proposed by the Mary and coworkers (1988)[33], where endogenous proteases other than thrombin, such as calpain, cathepsin C, or trypsin, can activate FXIIIa. In addition to the initial Arg-37-Gly-38 cleavage site, another Lys-513-Ser-514 cleavage site is present in FXIIIa[34]. The hydrolysis of FXIIIa by thrombin and/or other proteases leads to the formation of FXIIIa fragments of lower molecular weight, which remain partly active. This mechanism is thought to be an alternative way to activate intracellular FXIIIa, since thrombin can not penetrate the plasma membrane.

The tissue distribution and known functions of FXIIIa are relatively well documented. Its established physiological function in blood coagulation is based on its affinity for the substrate $\alpha_2$-antiplasmin[35]. Under normal physiological circumstances in blood, plasmin ensures the correct dissolution of blood clots. $\alpha_2$-antiplasmin is an inhibitor of plasmin, but its activation requires the crosslinking activity of FXIIIa. During the blood coagulation cascade, fibrinolytic resistance is partly achieved when thrombin-activated FXIIIa forms covalent cross-links within $\alpha_2$-antiplasmin, to form fibrin[36]. In a synchronized reaction, the clotting event is achieved when fibrinogen is converted to fibrin by thrombin.

The roles of FXIIIa are however not restricted to the fibrinolytic system. It has been showed that actin and myosin act as FXIIIa substrates[37], suggesting its involvement in cytoskeletal organization. FXIIIa is also implicated in cell adhesion and ECM assembly processes, as suggested by its adhesive substrate proteins FN and OPN (for review, see Muszbek et al, 1996[30]). Researches have also shown that the expression of the cellular form of FXIIIa is not restricted to platelets, monocytes and bone marrow cells, but extend to the skin, gut, placenta and chondocytes in growth plate cartilage[38]. Recently, Nurminskaya et al (2002)[39] have reported FXIIIa expression in differentiating chondrocytes. They have shown that FXIIIa expression correlates with chondrocyte differentiation and matrix mineralization. Interestingly, the group of Rosental (2001)[40] have demonstrated that FXIIIa is upregulated when pathological mineralization occurs, in this case, osteoarthritis. These findings suggest a function for FXIIIa in ECM mineralization, and suggest a broader range of physiological roles for FXIIIa than previously thought.

## 1.6 Transglutaminases in cartilage and bone

In 1993, Aeschlimann and colleagues[41] have demonstrated by immunohistochemistry that the expression of tTG correlates with chondrocyte differentiation and calcification of the cartilage matrix during endochondral bone formation. Subsequent immunohistochemical studies performed with an antibody raised against TGase crosslinks (ε-(γ-glutamyl)lysine crosslinks) confirmed the presence of TGase activity in cartilage and bone matrix[42]. However, it was concluded that these crosslinks could represent the end product of more than one isoform of transglutaminase. Consistent with this hypothesis, Nurminskaya et al[43] showed in 1998 that FXIIIa is upregulated in hypertrophic chondrocytes and that most of the enzyme activity is externalized to the ECM during the mineralization of the cartilage. Moreover, the FXIIIa transglutaminase has been identified as the major isoform of TGase present in avian cartilage[44]. Accumulating evidence suggested a role for tTG and FXIIIa in maturating cartilage, but the characterization of TGase expression by osteoblasts and its implication in bone formation remained unclear. In 2001, the group of Griffin[45] first demonstrated that tTG was expressed by human osteoblasts *in vitro* and that the enzyme is active. In parallel, Nurminskaya and colleages (2003)[46] demonstrated in chicken that tTG and FXIIIa secreted by hypertrophic chondrocytes regulate mineralization of ECM in a coculture system. They concluded that TGase is essential in mediating the differentiation and mineralization of osteoblasts.

Several lines of evidence further support the idea that transglutaminases, likely tTG and/or FXIIIa, play an important role in the ECM assembly and organization in bone, ultimately influencing mineralization. One predominant substrate for TGase crosslinking reaction in cartilage and bone is OPN. Osteopontin, as introduced previously, is believed to play a role in regulating ECM mineralization. In bone, TGase polymerizes OPN, as demonstrated by Kaartinen and coworkers[28]. This protein polymerization could result in the reorganization of charges in a local environment and contribute to the regulation of

mineral formation and growth[47]. Kaartinen and colleagues also provided evidence that the crosslinking of OPN by tTG is regulated by osteocalcin[48], another abundant bone NCP. In addition, Kaartinen and coworkers[28] provided direct *in vivo* evidence of tTG activity in rat bones. However, the exact function(s) of TGase in bone remain unknown. To date, only one human osteoblast-like cell line has been studied in terms of tTG expression (Heath et al, 2001)[14]. FXIIIa expression has been extensively studied in cartilage, but there is a lack of data regarding its distribution in bone matrix and bone cells. The expression of other TGase isoforms in bone has not been investigated.

## 1.7 Osteoblast cell culture models

In order to assess the function of a protein in a specific tissue, the use of cell culture models is commonplace. The establishment of an appropriate bone cell culture model is complex, since bone is formed by subsequent differentiation steps of an original osteoprogenitor cell ( Figure 3). When choosing a cell culture model, it is important to show the homogeneity of the cell population, and to know the changes that occur with cell differentiation. Unfortunately, the molecular mechanisms underlying lineage commitment and progression are not yet fully understood.

The fate of cells isolated from bone marrow for primary cell cultures is sometimes difficult to track, and the resulting cell model often exhibits a mixed population of cells from a common origin (osteoblasts, adipocytes, fibroblasts, etc.). However, it is possible to restrict the cell population to a single lineage by controlling culture conditions[49]. Once a single lineage of osteoprogenitors has been established, further steps of osteoblast differentiation are more characterized. During their differentiation process, osteoblasts slow their proliferation and start expressing specific bone formation marker genes, such as osteocalcin and alkaline phosphatase. Osteoblasts produce a collagen-rich matrix in which they become embedded. Mature osteoblasts in culture are recognized by their ability to mineralize the ECM in a manner similar to bone mineralization. The availability of a well-characterized model to study the function of a protein during the different stages of osteoblast differentiation is quite limited. The various immortalized

bone cell lines available are generally heterogeneous in their gene expression profile. Furthermore, transformed cell lines do not display normal replication rates, differentiation and cell cycle phases[50]. The use of primary cells as a model allows the tracking of osteoblast differentiation, however, this model is often limited by the presence of other cell lineage and by a time-limiting frame for experiments. In addition, subcultivation of primary cells may results in the dedifferentiation of the osteoblasts, marked by a loss of osteoblast markers and phenotype[51].

The MC3T3-E1 osteoblast-like cell line was established from newborn mouse calvaria[52]. MC3T3-E1 cells were first isolated by Kodoma and coworkers (1981)[52] and further subcloned by Wang and colleagues in 1999[53]. Upon different treatments, MC3T3-E1 cells undergo stages of either proliferation, differentiation and mineralization (Figure 4). Although the stages of differentiation and mineralization are progressive, each stage is characterized by the expression of typical gene markers associated with a distinct phenotype. The different genes and transcription factors involved in the developmental sequence of MC3T3-E1 cells have been studied in detail by the group of Seth (2000)[54-55].

For most cells, the stage of proliferation is characterized by the expression of cell cycle and cell growth genes. The stage of differentiation for osteoblasts, induced by treatment with ascorbic acid (AA), is marked by the expression of genes related to the formation and the modification of the ECM, such as collagen type I (COLLI) and alkaline phosphatase (ALP). The mineralization stage is characterized by the expression of genes such as OPN, BSP, and OCN, which correspond to mineral-binding proteins.

The detailed temporal sequence of osteoblast marker gene expression in MC3T3-E1 cells has also been studied by Franceschi[56]. In addition to a detailed genetic analysis, they have further characterized the differentiation phenotype of MC3T3-E1 cells and their requirement for AA. Ascorbic acid is an essential cofactor for the enzyme lysyl oxidase, which aids in the covalent assembly of collagen fibrils. It stimulates the hydroxylation and processing of collagen type I, therefore increasing collagen matrix formation. The formation of a collagen-rich matrix is essential for the further expression of the osteoblast phenotype. This requirement for AA is not restricted to the MC3T3-E1 lineage, but also applies to a variety of other osteoblast cell culture models such as primary cultures of fetal rat calvaria cells[57], chicken preosteoblasts, many osteoblast-like cell lines such as

UMR20 and RCT1, and the bone marrow stromal ST2 cell line[58]. However, transformed cell line such as ROS17/2.8 synthesize a collagen matrix and exhibit osteoblast markers expression without the addition of AA[59]. The detailed role of AA in osteoblast differentiation has been studied by Xiao and colleagues (1997)[62] and AA appears to induce the promoter of the osteocalcin gene. More specifically, AA is thought to target an osteoblast-specific element present on the osteocalcin gene promoter. However, the same study has demonstrated that collagen type I synthesis precedes and is required for the induction of OCN expression by AA. Thus, AA may have multiple roles in the differentiation of osteoblasts, especially in MC3T3-E1 cells.

MC3T3-E1 cells are a well-characterized and established cell culture model available to study osteoblast differentiation and matrix maturation. Similarities with the temporal sequence of *in vivo* bone formation support its biological relevance. However, like every *in vitro* system, the MC3T3-E1 cell line has limitations. Since MC3T3-E1 are not immortalized, their lifespan is limited to a certain number of passages. The replicative senescence of MC3T3-E1 cells has been studied by the group of Miller (2001)[60]. They found that above passage 60, cells show a significantly reduced proliferation rate. They also demonstrated that between passages 20 and 60, MC3T3-E1 cells progressively alter their morphology and display an enlarged size. This is accompanied by the alteration of their gene expression pattern. All these signs are characteristic of senescent cells. Thus, it has been recommanded to use cells with a passage number less than 20 for experiments.

Another limitation encountered in using MC3T3-E1 cells came with the observation by several groups that MC3T3-E1 cells show heterogenous phenotype colonies within the parent cell line[61,,62,63]. The source or the reason for this phenotype plasticity is still unknown, but it has been speculated that it could result from chromosomal instability, or it could reflect a flexible property of osteoblasts. In addition, it is notable that preosteoblasts and osteoblasts show an heterogenous phenotype *in vivo*, depending on their position in the calvariae bone[38]. Thus, a differential expression pattern may reflect small variations in the cellular microenvironment associated with cell culture. To resolve this problem of MC3T3-E1 cell heterogeneity, Wang and collegues (1999)[53] isolated and characterized subclones from MC3T3-E1 cells. The subclones have

been divided in two main categories: mineralizing (clones # 4, 8, 11, **14**, 26) and non-mineralizing (clones # 17, 20, 24, 30, 35) . All of them have been characterized in terms of collagen formation, DNA content, expression of differentiation markers and mineralization. Moreover, subclones # 4, **14** and 26 have been tested positive for their *in vivo* osteogenesis potential Based on the data arising from this study, we opted to use the cell line MC3T3-E1 subclone number 14 (MC3T3-E1/C14) to do the experiments presented in this work. These cells present a full differentiation program, and produce a collagen-rich matrix that mineralizes. Also, during this differentiation pathway, abundant NCPs are expressed and assemble into the mineralized ECM.

# 2. AIMS OF THE STUDY

In order to determine which TGases are expressed by osteoblasts and are active in bone formation, we have investigated tTG and FXIIIa expression and activity during osteoblast differentiation, matrix production and mineralization *in vitro* using the MC3T3-E1/C14 cell culture model. To characterize TGase expression during osteoblast maturation and matrix mineralization, we aimed to:

- characterize MC3T3-E1/C14 osteoblast differentiation and matrix maturation steps.
- study different TGase isoforms expression by osteoblasts using RT-PCR.
- study TGase activity during osteoblast maturation.
- study TGase expression at the protein level by Western blotting and by immunohistochemistry techniques.

Ultimately, this work will help to elucidate the function of TGases in bone.

# 3. MATERIALS AND METHODS

## 3.1 Cell cultures and treatments

MC3T3-E1 preosteoblast cells (subclone C14) were a generous gift from Dr. Renny T. Franceschi (University of Michigan School of Medecine, Ann Arbor, Michigan, U.S.A.). Cells were routinely grown in modified alpha minimum essential medium (α-MEM with L-glutamine, with sodium bicarbonate at 2.2 g/L, without ascorbic acid, without deoxyribonucleosides and without ribonucleosides, Gibco,Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% penicillin-streptomycin antibiotics (Gibco). Cells were passaged every 5-7 days and were not used beyond passage 15. For studies characterizing osteoblast differentiation and mineralization, MC3T3-E1 cells were plated at an initial density of 50,000 cells/cm$^2$ in 35, 60 or 100 mm tissue culture dishes. The following day (day 0) the growth media was replaced with medium suitable for the desired experimental condition. Differentiation and mineralization were induced respectively by the addition of 50 µg/ml ascorbic acid (AA) (Sigma, St-Louis, MO) or by the addition of 50 µg/ml AA plus 10 mM β-glycerolphosphate (βGP) (Sigma). Thereafter, cells were supplemented with respective experimental conditions (AA or AA+ βGP) from freshly prepared stocks when the growth medium was replaced every second day, for a total period of 12 days (Figure 5). All cell cultures were maintained in a humidified atmosphere at 37°C and at 5% $CO_2$ in a cell incubator.

## 3.2 Biochemical assays

### 3.2.1 Collagen staining and quantification by sirius red

Preosteoblast cell differentiation into matrix-producing osteoblasts was assessed by measuring collagen production. Collagen was visualized and quantified with sirius red staining as previously described[64]. Briefly, cells were seeded in 35 mm dishes (Costar, Cambridge, MA), subjected to different experimental conditions, as described in Figure 6,

and stained at different time points. Cell layers were rinsed 3 times with PBS before they were fixed with Bouin's fluid for one hour. The fixation fluid was removed and the culture dishes were rinsed and left in distilled water for 15 minutes. The water was removed and the culture dishes were air dried. The sirius red dye reagent (Sigma) was added and incubated for 1 h under agitation. The dye solution was removed and the cell layer was washed with 0.01 N hydrochloric acid (HCl) to remove all non-bound dye. Cell morphology was photodocumented before dissolving the stain. The stained material was dissolved in 0.1 N sodium hydroxide for quantification. The dye solution was transferred to microtiter plate and the optical density (OD) measured at 562 nm. Soluble calf skin collagen type 1 (Sigma), immobilized onto cell culture dishes by an overnight incubation and stained with sirius red as described, was used for the standard curve.

### 3.2.2 von Kossa staining

*In vitro* mineralization of cells plated in 35 mm dishes, grown in different experimental conditions, was visualized by von Kossa staining after 1,2,4,6,8,10 and 12 days of treatment. The cell layer was rinsed twice with phosphate-buffered saline (PBS) and fixed with 100% ethanol for 15 minutes at 37°C. After, the cell layer was rehydrated by consecutive washes with 95%, 80%, 50%, 20% and 0% ethanol and stained with 5% silver nitrate for one hour at 37°C. The stain was removed; the cell layer was rinsed with water, and then exposed to light for 30 minutes. Cells were then air dried and the resulting staining was photodocumented.

### 3.2.3 Calcium and phosphate assays

For measurement of calcium and phosphate, cell layers grown in 100 mm culture dishes were extracted overnight with 15% trichloroacetic acid and cell debris was removed by centrifugation.

*Calcium*: Supernatants were assayed for calcium using a calcium kit (Sigma) according to manufacturer's procedure.

*Phosphate*: Phosphate was measured using the method of Heinonen and Lahti[65]. Briefly, 250 µl of the supernatant was mixed with 2 ml of AMM reagent solution (50%

acetone, 25% sulfuric acid, 25% ammonium molybdate), followed by the addition of 200 µl of citric acid. The OD was measured at 355 nm and known concentrations of inorganic phosphate (sodium phosphate monobasic, Sigma) were used as standards.

## *3.3 Activity assays*

### 3.3.1 Alkaline phosphatase (ALP) activity assay

Cells seeded in 35 mm culture dishes and cultured under different growth conditions were rinsed twice with ice-cold PBS. On ice, 750 µl of harvest buffer containing 10 mM Tris pH 7.4, 0.2% IGEPAL (Sigma) and 2 mM PMSF was added to the cells. The cell layer was scraped off using a cell scraper (Costar) and transferred to a sterile 15 ml conical tube. The culture dish was rinsed with an additional 750 µl of harvest buffer, and transferred to the tube. The cell suspension volume was made complete to 2 ml with the harvest buffer. Cells were then homogenized with a sonicator and centrifuged at 2,000 RPM for 10 minutes at 4°C to separate cell debris. The supernatant was used for the ALP assay. The ALP activity was determined using Sigma's kit with *p*-nitrophenylphosphate (pNPP) as a substrate.

### 3.3.2 Dot blot transglutaminase activity assay

This activity assay was carried out in microtubes. Cell extracts tested for FXIIIa activity were first digested with 200 mU of thrombin for 15 minutes at 37°C in a reaction buffer containing 25 µg or 50 µg of total protein (from cell extracts), Tris-buffered saline (TBS) pH 8.0, and 3 mM calcium chloride. This reaction buffer was adjusted to contain 5 mM of biotinylated primary amine (bPA) (Pierce, Rockford, IL, USA)), 1 mM dithiothreitol (DTT) (Sigma) and 1 µg of fibronectin (Sigma) was added as a TGase substrate. The total volume of each sample was 200 µl. Samples were mixed and incubated at 37°C for 2 h (TGase activity assay). The labeling mechanism of the TGase reaction is illustrated in Figure 5. The reaction product was dotted on a polyvinylidene difluoride PVDF membrane using a dot blot apparatus (Bio-Rad) according to the

manufacturer's instructions. The membrane was blocked with 5% non-fat milk powder in Tris-buffered saline-Tween (TBS-T) for 1 h under agitation, at room temperature. Membrane was then incubated with horseradish peroxidase-conjugated extravidin antibody (Sigma) diluted 1:25000 in TBS-T. Dot blots were visualized using an Enhanced Chemiluminescence (ECL Plus kit) (Amersham) according to the manufacturer's instructions.

## 3.4 DNA isolation and quantification

Cells were grown in 35, 60 and 100 mm culture dishes and subjected to different experimental conditions. At the indicated time points (see Figure 6), medium was removed, the cell layer was rinsed twice with PBS and total DNA was isolated from the cells according to the method described by Laird et al[66] which included three steps. First, the lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCL, 100 µg Proteinase K/ml) was added to the cells directly in the culture dish. Digestion was completed by an overnight incubation at 37°C under agitation. Second, one volume of 100% isopropanol was added to the lysates and the samples were swirled until the DNA precipitation was completed (30 minutes to 1 h). Third, the DNA was recovered by lifting the aggregated white precipitate using a pipette tip. The DNA was dispersed in an eppendorf tube containing 500 µl of 10 mM Tris, 0.1 mM EDTA, pH 7.5. Complete dissolution of the DNA was achieved by several hours of agitation at 37°C.

DNA was quantified using the diphenylamine assay according to the methods of Schneider[67]. The whole sample (500 µl) was mixed with 500 µl of 0.8 M perchloric acid (PCA), and incubated for at least 15 minutes on ice. Samples were centrifuged at 2000 RPM for 10 minutes at 4°C. The supernatant was removed and the samples were washed twice with 1 ml of ice-cold 0.4 M PCA. After the last wash, samples were resuspended by vortexing in 1 ml of 0.4 M PCA, and boiled for 30 minutes. Samples were cooled down to room temperature and centrifuged at 2000 RPM for 10 minutes at 4°C. The supernatant was split into two fresh tubes (duplicate, 500 µl/tube). One milliliter of fresh reagent solution (1.5% sulfuric acid in glacial acetic acid, supplemented with 80 µg/ml acetaldehyde and 15 mg/ml diphenylamine) was added to every 500 µl sample. Samples

were covered with aluminum foil and incubated in the dark for 14-16 hours. Samples were transferred to plastic cuvettes (Sarstedt) and absorbance was measured at 600 nm. Various concentrations (0-200 µg) of calf thymus DNA (Sigma) were used to establish a standard curve.

## 3.5 RNA isolation

For RNA isolation, cells were seeded in 35 mm dishes (Costar, Corning, NY) in duplicate and subjected to different experimental conditions. At the indicated time points (see Figure 5), medium was removed, the cell layer was rinsed twice with PBS and total RNA was isolated from the cells using Trizol reagent (Gibco) following the manufacturer's instructions. RNA concentration was determined by comparaison with mouse liver RNA as standard (Ambion, Austin, TX) run on a 1% denaturing agarose gel.

## 3.6 Reverse transcription-polymerase chain reaction (RT-PCR)

For RT-PCR analysis, 1 µg of total RNA for each sample was reverse transcribed and amplified using SuperScript one-step RT-PCR with Platinum *Taq* (Invitrogen Corporation, Carlsbad, CA) using 100 pmole of each primer in a total reaction volume of 50 µl. The amplification parameters are described in Table 3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. RT-PCR reactions without an RNA template were used as negative controls to ensure that the detected bands were not the product of contaminating RNA. Five µl of every RT-PCR product was separated by electrophoresis on 2% agarose gels stained with ethidium bromide. Also, the presence of contaminating DNA was assessed by PCR experiments carried on the isolated RNA using iTaq DNA Polymerase and dNTP Mix (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's specification. This test demonstrated that the isolated RNA was pure.

**Table 3. List of primers and experimental conditions used for each RT-PCR reaction**. Primer sequences and RT-PCR conditions were chosen from previously published work or designed using Primer3 software available on the website www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. The melting temperature (Tm) of the primers was determined using the Oligo calculator from the website www.bs.jhmi.edu/proteinfacility/oligos/oligohome.html. Primers were purchased from Invitrogen (Burlington, ON, Canada) or were prepared at McGill's Sheldon Biotechnology Center (McGill University, Montreal, QC, Canada).

| Gene Name | Reference | Sequence Name | Sequence (5'---- 3') | Length | Tm | Concentration (picomole/µl) | Product length (bp) | Annealing T° (°C) | Elongation Time (sec) | Number of cycles |
|---|---|---|---|---|---|---|---|---|---|---|
| Alkaline Phosphatase | Chung et al, Biochem Biophys Res Com (1999) 265:246-251[68] | ALP-1 | 5' CCAAGACGTACAACACCAACGC 3' | 22 | 57 | 208,5 | 474 | 55 | 30 | 30 |
| | | ALP-2 | 5' AAATGCTGATGAGGTCCAGGC 3' | 21 | 54 | 231,0 | | | | |
| Bone Sialoprotein | Saito et al, J Cell Sci (2002) 112:4191-4200[69] | BSP-1 | 5' AACAATCCGTGCCACTCA 3' | 18 | 48 | 262,5 | 1048 | 58 | 45 | 24 |
| | | BSP-2 | 5' GGAGGGGGCTTCACTGAT 3' | 18 | 53 | 401,4 | | | | |
| Collagen Type I | Ehara et al, Biomaterials (2003) 24 :831-836[70] | COLL-1 | 5' GAGGCATAAAGGGTCATCGTGG 3' | 22 | 57 | 339,2 | 739 | 55 | 45 | 30 |
| | | COLL-2 | 5' CATTAGGCGCAGGAAGGTCAGC 3' | 22 | 59 | 192,6 | | | | |
| Factor XIIIa | XM138580 (GenBank accession number) | FXIIIa-1 | 5' CCAGATTGACTTCAACCGTCCC 3' | 22 | 57 | 145,0 | 708 | 55 | 45 | 30 |
| | | FXIIIa-2 | 5' GACACCAGCAAAAACCCAACACTGG 3' | 25 | 59 | 238,0 | | | | |
| Factor XIIIb | Nonaka et al, Genomics(1993) 15 : 535-542[71] | FXIIIb-1 | 5' ACCCGAAGACAAATGGA 3' | 17 | 45 | 100,0 | 103 | 55 | 30 | 30 |
| | | FXIIIb-2 | 5' TAAAGGCTCAGAACACT 3' | 17 | 42 | 100,0 | | | | |
| Glyceraldehyde-3-phosphate dehydrogenase | NM008084 (GenBank accession number) | GAPDH-1 | 5' TGGCAAAGTGGAGATTGTTG 3' | 20 | 60 | 216,0 | 600 | 55 | 45 | 35 |
| | | GAPDH-2 | 5' TTCAGCTCTGGGATGACCTT 3' | 20 | 60 | 254,0 | | | | |
| Osteocalcin | Ehara et al, Biomaterials (2003) 24 :831-836 | OC-1 | 5' CTGGCCCTGGCTGCGCTCTGT 3' | 21 | 62 | 238,0 | 267 | 60 | 20 | 35 |
| | | OC-2 | 5' GGTCCTAAATAGTGATACCGTAGATGC 3' | 28 | 60 | 488,0 | | | | |
| Osteopontin | Ehara et al, Biomaterials (2003) 24 :831-836 | OPN-1 | 5' CTGCTAGTACACAAGCAGACA 3' | 21 | 52 | 154,8 | 488 | 45 | 30 | 35 |
| | | OPN-2 | 5' CATGAGAAATTCGGAATTTCAG 3' | 22 | 49 | 306,3 | | | | |
| Tissue Transglutaminase (TG2, tTG) | Citron et al, Neurochem Int (2000) 37 : 337-349[72] | tTG-P1 | 5' ATACAGGGGATCGGAAAGTG 3' | 20 | 49 | 96,9 | 455 | 55 | 30 | 35 |
| | | tTG-P2 | 5' GGCCAACCACCTGAACAAAC 3' | 20 | 55 | 70,0 | | | | |
| Transglutaminase Type 1 (TG1) | AF186373 (GenBank accession number) | TG1-1 | 5' TGAGTCCTCTGATCGCATTG 3' | 20 | 60 | 193,0 | 751 | 55 | 45 | 30 |
| | | TG1-2 | 5' TGTGTCGTGTGCAGAGTTGA 3' | 20 | 60 | 166,0 | | | | |
| Epidermal-type transglutaminase (TG3) | Hitomi et al, Int J Biochem Cell Biol (IJBCB) (2001) 33 : 491-498[73] | TG3-1 | 5' TGGCAGTAGGCAAAGAAGTC 3' | 20 | 52 | 120,0 | 360 | 56 | 30 | 30 |
| | | TG3-2 | 5' ACGTTCACAGGCTTCCGCAC 3' | 20 | 56 | 119,0 | | | | |
| Transglutaminase Type 5 (TG5) | Candi et al, J Biol Chem (2001) 276 : 35014-35023[74] | TG5-F2 | 5' GTGCTGGCTTTGGTGGTGGT 3' | 20 | 56 | 100,0 | 206 | 58 | 30 | 31 |
| | | TG5-R4 | 5' GGGACTGTAGTCTTTGATCTCACTGG 3' | 26 | 60 | 100,0 | | | | |

## 3.7 Western blotting of cell extracts

### 3.7.1 Preparation of cell extracts

Initially, cells were plated in 100 mm culture dishes and subjected to different experimental conditions. At the indicated time points, medium was removed, the cell layer was rinsed twice with PBS and then 500 µl of harvesting buffer was added on ice. The cells and the ECM were scraped using a tissue culture scraper (Costar), placed into an Eppendorf tube on ice, and homogenized using a sonicator. Two different kinds of harvest buffer were used: Buffer A and Buffer B (see below).

*Buffer A*: Buffer A consisted of 0.25 M sucrose (to stabilize the enzyme), 10 mM Tris pH 7.4 and 2 mM of protease inhibitor phenylmethylsulfonyl fluoride (PMSF), all obtained from Sigma. This buffer does not demineralize the cells but retains enzyme activity. After homogenization, samples harvested with Buffer B were centrifuged at 14,000 RPM, 15 minutes and 4°C to remove cell debris. The supernatant was transferred to a fresh tube and used for analysis.

*Buffer B*: Buffer B consisted of 0.5 M EDTA pH 7.4 (to demineralize the cell cultures), 0.25 M sucrose, 10 mM Tris pH 7.4 and 2 mM of PMSF. Buffer B allows the release mineral-bound protein. Samples harvested with Buffer B were treated as follows: after homogenization, samples were extracted overnight on a rotator, at 4°C. Samples were then centrifuged at 14,000 RPM for 15 minutes at 4°C to remove cell debris. The supernatant was transferred to a dialysis cassette (Slide-a-Lyzer, Pierce, Rockford, IL) and samples were dialysed overnight against 5 mM ammonium bicarbonate pH 8.0 at 4°C. The dialyzed samples are transferred to fresh tubes and used for analysis. Samples were stored at -20°C.

### 3.7.2 Protein assay

The protein concentration of the samples was determined using the bicinchonic acid (BCA) protein assay (Pierce) following manufacturer's instructions.

### 3.7.3 Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and Western blotting

Using the results from the BCA protein assay, 50 µg or 100 µg of total protein of each cell extract and positive controls were separated on 10% SDS-polyacrylamide gels under reducing conditions and subsequently transferred onto PVDF membranes (Bio-Rad) for 1 h, using 100 volts at 4°C. Western blotting procedures were followed as previously described[28]. Briefly, membranes were blocked with 5% nonfat milk powder in TBS-T overnight at 4°C. Membranes were then incubated with primary antibodies (Table 4) for 2 h at room temperature at a 1:500 or 1:1000 dilution in TBS-T. Membranes were washed 5 times for 5 minutes with TBS-T and then incubated for 1 h with corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) (Amersham Bioscience, Piscataway, NJ). Bands were visualized using an ECL Plus kit (Amersham) according to the manufacturer's instructions.

**Table 4. Antibodies used for Western blotting**. All secondary antibodies were purchased from Amersham Bioscience, Piscataway, NJ, USA. tTG, tissue transglutaminase; OPN, osteopontin; FXIIIa, factor XIIIa; HRP, horseradish peroxidase.

| Primary Antibody | Source | Secondary Antibody |
|---|---|---|
| Mouse anti-tTG Cub7402 | Neomarkers, Union City, CA | Anti-mouse HRP conjugated |
| Anti-β-actin | Sigma, St-Louis, MO, USA | Anti-rabbit HRP conjugated |
| Anti-human OPN | Dr. Larry W. Fisher, NIH/NIDCR, Bethesda, MD, USA | Anti-rabbit HRP conjugated |
| Anti-FXIIIa Ab-1 AC-1A1 | Neomarkers, Union City, CA | Anti-rabbit HRP conjugated |

## 3.8 Immunohistochemistry on paraffin sections

MC3T3-E1 cells were grown for 6 and 12 days in 35 mm culture dishes and were subjected to different experimental conditions. Cell layers were rinsed twice with PBS before the cells and the ECM were scraped using a tissue culture scraper (Costar) and placed into a glass tube. Cell layers were immediately fixed by immersion into periodate/lysine/paraformaldehyde (PLP) fixative for 2 hours at room temperature. The fixative was removed; the cell layer was rinsed with PBS and embedded in paraffin. Sections of 5 µm thickness were cut and processed for immunohistochemistry. Dewaxed sections were rehydrated with graded ethanol solutions. For some experiments, sections were decalcified for 30 minutes in a wet chamber with a 4.13% (w/v) EDTA solution at pH 7.4. Sections were subsequently incubated overnight with the primary antibody diluted 1:50 or 1:200 in TBS-T containing 5% serum and 0.2% (w/v) BSA (Table 5). As a negative control, sections were incubated using the same conditions with the omission of the primary antibody. Sections were washed once with TBS-T supplemented with 2.5% (w/v) sodium chloride and 0.05% (v/v) Tween-20 for 10 minutes, followed by 2 washes of 5 minutes with TBS-T. Sections were then incubated for 45 minutes in a wet chamber, with corresponding biotinylated secondary antibody (Table 5) diluted 1:200 in TBS-T containing 0.2% BSA.

**Table 5. Antibodies used for immunohistochemistry**. All secondary antibodies were purchased from Sigma, St-Louis, MO, USA. tTG, tissue transglutaminase; OPN, osteopontin; FXIIIa, factor XIIIa.

| Primary Antibody | Source | Serum | Secondary Antibody |
|---|---|---|---|
| Goat anti-tTG | Upstate Biotechnology, Lake Placid, NY, USA | Rabbit | Anti-goat immunoglobulins biotin conjugated |
| Anti-isopeptide | CovaLab, Lyon, France | Goat | Anti-rabbit immunoglobulins biotin conjugated |
| Anti-human OPN | Dr. Larry W. Fisher, NIH/NIDCR, Bethesda, MD, USA | Goat | Anti-rabbit immunoglobulins biotin conjugated |
| Rabbit anti-FXIIIa | Chemicon, Temecula, CA, USA | Goat | Anti-rabbit immunoglobulins biotin conjugated |

After another series of washing steps, alkaline phosphatase was linked to the biotinylated secondary antibody using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. Color development was achieved with Fast Red TR/Naphtol ALP substrate (Sigma). The reaction was stopped by immersing the sections in distilled water. Sections were counterstained with filtered methyl green (Vector Laboratories), and mounted under coverslips using Kaiser's jelly (13 g gelatin, 60 ml distilled water, 70 ml glycerol, 250 ul phenol).

## 3.9 Embedding of cells in acrylic resin and von Kossa staining of sections

Cells were grown in 35 mm culture dishes under different experimental conditions and harvested after 6 and 12 days. At these time points, the cell layer was rinsed with PBS and harvested on ice using a cell scraper. Cells were transfered to a glass bottle and fixed with 4% paraformaldehyde, 0.1% glutaraldehyde for one hour, at room temperature, on a rotator. Before dehydration, fixative was removed and the cells were rinsed twice with PBS. Cells were dehydrated with graded ethanol solutions. Dehydrated cells were incubated in a mixture of 50% ethanol, 50% London white acrylic resin (LRW) (Electron Microscopy Science, Fort Washington, PA) for 3 h at 4°C, on a rotator, and then transferred overnight to a mixture of 25% ethanol, 75% LRW. The next day, cells were incubated in 100% LRW for 4 hours at 4°C, on a rotator. Cells were put into flattened, gelatin capsules filled with LRW and incubated in a 60°C oven for 48 h to polymerize the resin. Sections of 1 μm thickness were cut and stained with von Kossa reagent (4% silver nitrate) for 30 minutes. Sections were counterstained with 1% toluidine blue and mounted under coverslips using LRW resin.

## 3.10 X-Ray diffraction

Mineral from 20 day-old MC3T3-E1 cultures grown in the presence of AA and β-glycerol phosphate was analyzed by X-ray diffraction (XRD) using a 12kW rotating anode Rigaku D/Max 2400 automated diffractometer equipped with a graphite diffracted-

beam monochromator (Cu$K\alpha$ radiation) and a theta-compensating slit assembly. Cells scraped from 100 mm dishes grown for 20 days under mineralizing conditions and originally frozen were prepared for XRD analysis by mounting on glass coverslips using one of the following two methods: (i) cell layers were transferred with a pipette onto the center of a glass coverslip, covered and pressed with a Mylar sheet to evenly spread the samples across the coverslip, and then dried in a desiccator for several hours, or (ii) cells were freeze-dried, pulverized into a fine powder, and then mixed in 50 µl of reagent-grade alcohol as suspension. The suspension was transferred with a pipette onto the center of a glass cover slip and air dried. The XRD patterns were acquired with a variable slit assembly and the following analytical conditions: operating voltage of 40 kV, beam current of 160 mA, step-size of 0.02 2θ, counting time of 1 second per step, and a scanning range of 20 to 60 degree 2θ.

## 3.11 Statistical analysis

All statistical analyses were performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Unless indicated otherwise, error bars represent SEM of three independent expetiments. Statistical significance was assessed by Student's *t*-test available in the Microsoft Excel software.

# 4. RESULTS

## *4.1 Characterization of MC3T3-E1 cells*

### 4.1.1 Collagen matrix production

Undifferentiated osteoblasts are characterized by active replication and rapid cell growth. MC3T3-E1/C14 cell proliferation was characterized by measuring the DNA content at different time points of undifferenting (untreated) and differentiating (AA and AA+ βGP treated) cultures (Figure 7). Untreated cells had a higher rate of proliferation than AA-treated cells, reflected by a higher DNA content. The DNA content of the cells peaked at day 8, and then reached a plateau. The plateau in proliferation observed probably reflects the stages where the cells can no longer proliferate due to contact inhibition. The DNA content of the cells was thereafter used to normalize data.

It is well established that MC3T3-E1/C14 cells produce a collagen-rich matrix when subjected to AA treatment. The deposition of a collagen matrix is a key step in the differentiation of preosteoblastic cells to mature osteoblasts. We characterized the ability of the MC3T3-E1/C14 cell culture system to produce a collagen matrix, and thus differentiated function. This was performed by sirius red staining. Figure 8 shows an increasing red staining for AA-treated cultures (AA and AA + βGP) relative to the number of days in culture. The corresponding quantitative estimation of collagen was obtained by elution of the stain from the culture dishes as shown in Figure 9. The addition of AA to the cell cultures induces collagen formation, detectable as early as day 1 after the AA treatment. Thereafter, the collagen amount accumulates to form a thick matrix. Untreated cultures show no collagen production.

Collagen type I accounts for 20% of the total protein production by mature osteoblasts. To further characterize the collagen production of MC3T3-E1/C14 cells, we analyzed their collagen type I mRNA expression by RT-PCR (Figure 10). It was observed that collagen type I gene expression by MC3T3-E1 cells was relatively constant troughout the time spent in culture, and that this expression was independent of the treatment. Interestingly, untreated cells seemed to express collagen type I at comparable

levels to the AA-treated cells. However, collagen protein secretion was observed only in the AA-treated cultures, as demonstrated by sirius red staining (Figures 8 and 9). Similar levels of type I collagen mRNA expression by immature osteoblasts have been observed previously by Quarles (1992)[50] who also showed minimal collagen accumulation into the ECM. Therefore, it is likely that despite a high rate of collagen mRNA expression, immature (untreated) osteoblasts fail to effectively process collagen, as proposed earlier by the group of Quarles. Taken together, these results show that collagen accumulation into ECM is dependant upon AA supplementation.

### 4.1.2 Mineralization of the extracellular matrix

Mineralization of the ECM represents a final stage of osteoblast differentiation and matrix maturation. We first characterized mineral deposition by von Kossa staining of MC3T3-E1/C14 cell cultures. As shown in Figure 12, visible mineralization occurs in the presence of AA and βGP supplemented media starting at day 8, although small zones of mineral were sometimes observed at day 6. The accumulation of mineral is stimulated by the addition of β-glycerol phosphate, which is cleaved to release inorganic phosphate by the enzyme alkaline phosphatase expressed by mature osteoblasts. Immature (untreated) cultures display no mineralization. Thus, the presence of an AA-induced matrix is absolutely necessary for mineralization. Mineral was also identified by von Kossa staining of plastic-embedded MC3T3-E1/C14 cell sections (Figure 13). Mineral preferentially deposited towards the dish surface side of the cultures, where the ECM is more mature.

Mineralization of mature MC3T3-E1/C14 cell cultures was further characterized by calcium and phosphate assays (Figure 14). In accordance with our previous results, an increase in cell layer-associated calcium and phosphate was first detectable at day 8 in AA- and βGP-supplemented cells, whereas cultures subjected to AA only did not show calcium or phosphate accumulation in the matrix. In order to verify the exact chemical composition of mineral in MC3T3-E1/C14 cell cultures, and to provide information on the type of mineral, we have analyzed it by X-ray diffraction as described in Materials and Methods. The diffraction pattern obtained demonstrated the presence of

hydroxyapatite (HA) crystals within the mineralizing cell cultures (Figure 15). The diffraction pattern of the mineral from MC3T3-E1/C14 cultures is similar to the diffraction pattern of mineral extracted from bone. Together with the chemicals and morphological assays, these results show that MC3T3-E1/C14 cultures produce mineral that resembles the HA crystals found in bone.

### 4.1.3 Osteoblast differentiation marker expression

One established marker of the mature osteoblast phenotype is an increase in alkaline phosphatase (ALP) expression[50,54]. ALP may also play a role in the regulation of osteoblast differentiation, as proposed by Aubin[75]. The precise function of ALP in bone cell biology remains undefined, but strong evidence points to ALP is involved in the mineralization process[76-77], where it releases phosphate ions to promote mineralization. In MC3T3-E1/C14 cell cultures, ALP releases phosphate ions from β-glycerol phosphate, commonly used to mineralize matrix *in vitro*. We have determined ALP activity in undifferentiating and differentiating MC3T3-E1/C14 cells at various time points. Figure 16 shows a low basal ALP activity in untreated cells, while treatment with AA increased the activity to a detectable level at day 6, after which the activity increased greatly during days 8, 10, and 12. Thus, AA is required for ALP activity induction. In AA-treated cells, ALP activity peaks at day 10, where it appears to reach a plateau level. Similarly, in cultures supplemented with AA and βGP, ALP activity was first detectable at day 6 and further increased at day 8, when mineralization commenced. However, ALP activity did not plateau at day 10 and continued to increase through day 12.

Mature osteoblasts are also characterized by their ability to express several bone matrix markers, such as the noncollagenous proteins bone sialoprotein (BSP), osteocalcin (OC), and osteopontin (OPN), as reviewed by Aubin[78]. MC3T3-E1/C14 cells mRNA levels were analyzed by RT-PCR for the following osteoblast marker proteins: BSP (Figure 17), OC (Figure 18), and OPN (Figure 19). When grown in the presence of AA, MC3T3-E1/C14 cells expressed high levels of mRNA for BSP, OC and OPN, with a trend of slight increase through days of culture. The addition of βGP did not significantly change the levels of mRNA expression of the tested genes. However, the untreated cells show undetectable or very low levels of mRNA expression for the above three genes.

These results are consistent with a previous report published by Wang et al (1999)[53]. Thus, the induction of osteoblast markers require AA. It is important to note that RT-PCR data presented in this thesis were obtained by scanning and comparing the intensity of RT-PCR bands. This method provides an indication of the relative presence of mRNA genes. Therefore, all RT-PCR data presented should be eventually validated by Northern blotting or Real-time PCR experiments.

Osteopontin, a substrate protein for the emzymatic action of tTG and FXIIIa, is also thought to play an important role in mineralization and cell adhesion processes (for review, see Sodek, 2000)[7]. The expression of OPN was further investigated by immunohistochemistry in mature MC3T3-E1/C14 cell cultures shown morphologically in Figure 20 by hematoxylin and eosin staining. Immunohistochemistry of mineralizing MC3T3-E1/C14 cells revealed a pattern of OPN protein expression different from the mRNA expression pattern. As shown in Figure 20, OPN protein was not present in the days preceeding mineralization (day 6) in AA- and βGP-supplemented cell cultures, althougth OPN mRNA was expressed at every time point (Figure 19). OPN protein was only detectable at later time points (day 12) in mineralizing cultures. Furthermore, OPN was expressed in patches that correlate with mineral deposition sites (see Figure 13). Western blots of OPN expression correlate with these data (see Figure 24) and are discussed later in conjunction with transglutaminase expression results (see section 4.2.3).

## *4.2 Transglutaminase expression in MC3T3-E1/C14 cells*

### 4.2.1 Detection of transglutaminase crosslink products

A large body of evidence suggests a role for transglutaminases in matrix mineralization and/or in ECM stabilization. Transglutaminase crosslinks are abundant in bone with a prominent localization to the newly formed bone osteoid and the pericellular matrix surrounding osteocytes, as demonstrated by Kaartinen and coworkers (2002)[28]. TGase crosslinks are also present in the mineralizing cartilage of the hypertrophic zone of long bones undergoing endochondral ossification (Aeschlimann et al, 1993)[19] as well as in mineralizing (osteoarthritic) articular cartilage (Rosental et al, 2001)[40]. We used an

antibody against isopeptide bonds to detect the presence of transglutaminase crosslink products in MC3T3-E1/C14 cell cultures. As shown in Figure 22, isopeptide bonds were readily detected in the ECM and the cells, independent of the treatment or the time of culture, although the precise compartment stained was difficult to ascertain.

### 4.2.2 Screening of transglutaminase isoforms expression

Among the nine TGase isoforms identified to date (Table 5), two have been proposed to be involved in bone biology, namely FXIIIa and tTG. However, tTG is the only isoform that has been studied in bone *in vivo* and *in vitro*. FXIIIa has been investigated in hypertrophic chondrocytes of the growth plate where it co-localizes with tTG, but its has not been detected in mature osteoblasts nor in bone. To determine which TGase isoforms were expressed by mineralizing MC3T3-E1/C14 cells, we analyzed MC3T3-E1/C14 mRNA by RT-PCR for the following transglutaminase isoforms: TG1, TG2 (tTG), TG3, TG5, and FXIIIa. The results of this analysis are presented in Figure 23.

Some isoforms (TG4, TG6, TG7 and Band 4.2) were not analyzed for the following reasons: (i) their cDNA sequence was not available, or (ii) the isoform has been demonstrated previously to be very specific to a certain tissue or organ. As shown in Figure 23, mineralizing MC3T3-E1/C14 cells expressed a constant, high level of tTG (TG2). The cells also expressed high levels of FXIIIa, the latter increasing in function over time during mineralization. MC3T3-E1/C14 cells did not express detectable levels of TGase isoforms TG1, TG3, and TG5, which have been tested with published primerss and the best possible experimental RT-PCR conditions. Lack of expression for these isoforms was anticipated given that they are mainly expressed in epidermal tissues (see Table 5).

### 4.2.3 Osteopontin expression and polymerization

Since OPN is a substrate protein for both TGase isoforms (Aeschlimann et al, 1996)[29] expressed by MC3T3-E1/C14 cells, namely tTG and FXIIIa, cell extracts were analyzed for the presence of OPN polymers. OPN has a high affinity for hydroxyapatite

*in vitro* as documented by Hunter[6], data which correlates with observations *in vivo* describing the presence of OPN at sites of tissue mineralization[79]. Also, results from the present work have demonstrated that OPN localization correlates with HA mineral patches in MC3T3-E1/C14 cultures. It has been shown that for OPN and other mineral-binding proteins, that mineral dissolution is required to release them from the tissue (Kaartinen et al, 2002)[28]. Therefore, we used a demineralizing harvest buffer containing 0.5 M EDTA, named *"Buffer B"* (see section 3.6.1), to extract OPN from cell cultures. Western blotting of cell extracts show that in untreated cells or in cells supplemented with AA only, OPN was produced at a very low, basal level (Figure 24). However, in mineralizing cells, OPN expression was clearly elevated at day 8, correlating exactly with the first time point where mineral deposition was detected in the cultures. At day 10, OPN expression further increased and we OPN polymers also became evident. At day 12, OPN polymers were clearly present. These data suggest that the presence of OPN polymers in mineralizing cultures are attributable to tTG and/or FXIIIa activity, supporting the hypothesis that transglutaminase may have an important function in mineralization.

### 4.2.4 Tissue transglutaminase expression and activity in MC3T3-E1/C14 cells

In light of previous results showing the presence of TGase crosslink products in cells and matrix, tTG and FXIIIa mRNA expression by osteoblasts, and OPN polymerization *in vitro*, we further investigated TGase enzyme activity and expression in MC3T3-E1/C14 cell culture model.

The tTG isoform was first analyzed, whose expression has been characterized in a human osteosarcoma cell line (Heath et al, 2001)[14] and in bone (Kaartinen et al, 2002)[28]. However, no data on its expression during osteoblast maturation is available. Levels of tTG mRNA expression in undifferentiating (untreated), differentiating (AA) or mineralizing (AA + βGP) MC3T3-E1/C14 cells were determined by RT-PCR. tTG mRNA was expressed at relatively constant, high levels with a slight trend (not

significant) towards increasing during time in culture. tTG mRNA expression in MC3T3-E1/C14 cells was not significantly changed by the addition of AA or βGP (Figure 25).

The expression pattern for tTG was reflected at the protein level by Western blot analysis of cell extracts (Figure 26). Consistently, tTG protein levels remained constant during time in culture, and they were not affected by treatment with AA or βGP. The tTG antigen in cell extracts is present in very low amounts (the blots shown have been exposed for 30 minutes compared to β-actin, exposed for 30 seconds). Thus, the level of protein detected does not reflect the apparent high levels of mRNA expressed. This may be explained by one or more of the following hypotheses: (i) all mRNA is not transcribed into a protein; (ii) tTG is secreted to the ECM, where it becomes incorporated in the collagen matrix, where it is difficult to extract; (iii) tTG protein has a high turnover rate, thus it is proteolyzed quickly and does not accumulate in cells or matrix.

Tissue transglutaminase antigen quantities observed in Western blots correlate with the tTG activity results (Figure 27). Tissue transglutaminase extracted from MC3T3-E1/C14 cells shows a constant, low level of activity that is not significantly affected by treatment with AA or βGP, however, activity showed a slight decrease throughout time in culture. Tissue transglutaminase likely gets incorporated into the collagenous ECM, where its activity could be affected by matrix/mineral association. Tissue transglutaminase protein in MC3T3-E1/C14 cell layers was further analyzed by immunohistochemistry. The results presented in Figure 28 reveal that tTG is homogeneously distributed throughout the cells and the matrix, with no obvious preferential accumulation at specific sites.

Two kinds of negative controls were performed to ensure that the observed immunostaining was specific for the tTG antigen examined. First, sections were processed with primary antibody omitted, but secondary antibody retained, and no staining was obtained, indicating that the labeling was associated with a specific IgG (Figure 38). To control for the specificity of the tTG antibody in recognizing its antigen, we incubated the sections with an unrelated antibody raised in the same animal species (rabbit) as the tTG antibody. We used an antibody against dentin phosphoprotein, an antigen found only in tooth dentin. The immunoreaction for this unrelated IgG in MC3T3-E1/C14 cell sections was negative (data not shown). Precise localization of

immunohistochemical staining in paraffin sections of cultured osteoblasts is challenging in that the cell/tissue layer is very thin, and cell culture integrity is difficult to maintain after scraping off the cell layers prior to embedding in paraffin. Attempts to refine the localization of tTG by direct staining of the cells in the dish were unsuccessful, most likely attributable to restrictions in penetration of the antibody into the multilayered cell culture rich in collagen. Attempts to localize tTG at the electron microscopy level were unsuccessful. Fluorescence microscopic studies are currently underway to further elaborate tTG localization.

### 4.2.5 FXIIIa expression and activity in MC3T3-E1/C14 cells

The results showing that mineralizing MC3T3-E1/C14 cells express FXIIIa transglutaminase is a very interesting finding, since this TGase isoform has not been reported for bone cells. To further characterize its expression, FXIIIa mRNA expression was analyzed in MC3T3-E1/C14 cells at different stages of differentiation. RT-PCR results presented in Figure 30 demonstrate a trend towards increasing levels of FXIIIa expression relative to the time of culture. MC3T3-E1/C14 cells undergoing mineralization (AA + βGP treatment), FXIIIa expression increased in a constant, stable manner, independent of how the cells were supplemented. However, this increase in mRNA expression was most evident in mineralizing cells. This might indicate that FXIIIa expression is influenced by the mineralization state of the cells. Therefore, we extracted the enzyme from cell harvests with two different types of buffer, one containing EDTA and the other one without EDTA (see section 3.6.1).

Prior to the analysis of FXIIIa by Western blotting, we tested several different FXIIIa antibodies with a series of positive controls. FXIIIa Ab-1, from Neomarkers (Union City, CA) was selected as a positive control on mouse plasma (Sigma). FXIIIa Ab-1 recognized a band at 52 kDa in mouse the plasma positive control (Figure 29). This band corresponds to the proteolyzed version of FXIIIa, as reported by many groups[22, 25, 80]. In human plasma used as a positive control, FXIIIa Ab-1 recognized a band at 76 kDa, which corresponds to the expected molecular weight of cleaved and activated FXIIIa[30].

Western blot analysis of cell extracts first showed a very strong positive reaction for a presumed FXIIIa band (Figures 31 and 32), which was unchanged as a function of time, treatment with AA and βGP, or the buffer used to extract proteins. These results were surprising, since FXIIIa mRNA expression levels vary with time and treatment of the cultures. Furthermore, a detailed molecular weight analysis of the band indicated a size of 37 kDa, which has never been characterized as a possible proteolytic product of FXIIIa, but could represent monomer form of this enzyme.

In order to further investigate FXIIIa activity in MC3T3/E1 cells, we performed a dot blot transglutaminase assay specific for FXIIIa activity whereby cell extracts are subjected to thrombin digestion as described in Materials and Methods. This treatment activates FXIIIa enzyme, which can be observed in transglutaminase assays as an increase in total TGase activity. Results from this assay (Figures 33, 34, 35) clearly show that cell extracts contain inactive transglutaminase FXIIIa that can further be activated by thrombin. Quantification of the dots intensity show that FXIIIa activity is substantially higher in EDTA-extracted cells. Association of FXIIIa protein with mineral in this case is not likely, since untreated cells show the highest FXIIIa activity. This result might indicate that EDTA protects FXIIIa activity, since FXIIIa can be non-proteotically activated by the presence of calcium ions. Therefore, the following focuses on the results from cells extracted with EDTA-containing buffer (Buffer B).

In contrast to the Western blot results, we found that FXIIIa-related activity shown in thrombin-digested samples significantly increased with time in culture. Interestingly, FXIIIa activity was higher in untreated, undifferentiating cells than in AA- and AA- plus βGP- treated cells, indicating that FXIIIa-related activity is influenced by AA treatment. This difference in activity could be acounted for by the fact that FXIIIa is secreted into the ECM and is trapped in the collagenous matrix, where the protein is more difficult to extract or is inactivated.

The expected molecular weight of inactive FXIIIa is around 80 kDa, and its thrombin-activated form is 76 kDa, which corresponds to the cleavage of the activating peptide of 4 kDa. The presence of bands corresponding to the inactive and activated forms of FXIIIa in cell extracts were investigated by Western blotting. Day 6 and 12 extracts from untreated cells, samples from which we have previously demonstrated the

highest FXIIIa activity, were chosen for further analysis. Prior to Western blotting, one set of samples was pre-digested with thrombin. As shown in Figure 36, two bands of low intensity at molecular weights of 81 and 77 kDa in undigested samples were clearly identified. Following thrombin digestion, the 81 kDa band faded to an undetectable level, and only the 77 kDa band remained.

These results suggest that the 81 kDa band likely corresponds to an inactive form of FXIIIa in cell extracts, and the 77 kDa band corresponds to the thrombin-activated form of FXIIIa. The 37 kDa band shown in Figures 31 and 32 remain unidentified. However, that band likely represents a variant form of FXIIIa, since the specificity of the antibody used has been demonstrated on mouse and human plasma (see Figure 29). It could represent a splicing variant of FXIIIa, or the result of a yet uncharacterized cleaveage product of FXIIIa. This 37 kDa band could also represent monomeric forms of FXIIIa subunits (dissociated 2 x A subunits), since it is approximately half the molecular weight of the 77 kDa homodimer FXIIIa. Interestingly, Lorand et al[81] reported that although it is generally assumed that FXIII subunits are held together by electrostatic and hydrophobic forces, it is not known whether the homodimeric association is constantly maintained.

Finally, FXIIIa expression in MC3T3-E1/C14 cells was localized by immunohistochemistry. In general, staining gave a strong signal (Figure 37). In untreated cells, FXIIIa expression was clearly polarized in MC3T3-E1/C14 cells. After treatment with AA, FXIIIa was incorporated into the ECM. This secretion into the matrix was more obvious at later time points (day 12), while FXIIIa expression remained mostly cellular and polarized at day 6, even after AA treatment.

# 5. DISCUSSION

## 5.1 MC3T3-E1/C14 cells as an *in vitro* model system to study the process of osteoblast differentiation

The overall aim of these studies was to investigate transglutaminase expression and activity during osteoblast differentiation. We used a well-established *in vitro* cell culture model, the preosteoblast MC3T3-E1/C14 cell line, that is known to undergo differentiation, maturation and mineralization when supplemented with ascorbic acid and β-glycerolphosphate. We have demonstrated that in our hands, the MC3T3-E1 subclone C14 cell culture model behaves as earlier reported (Kodoma et al, 1981[52]; Franceschi et al, 1992[56]; Quarles et al, 1992[50]; Wang et al, 1999[53]; Vary et al, 2000[54]).

Upon treatment with ascorbic acid, MC3T3-E1/C14 cells secrete an abundant collagenous matrix. To induce this differentiation process, we used ascorbic acid at a concentration of 50 μg/ml. Although the normal physiological concentration of ascorbic acid in human plasma ranges from 5 to 10 μg/ml, our results suggest that the effect of ascorbic acid on MC3T3-E1/C14 cells mimics the physiological effects of this vitamin. The results of our experiments indicated that ascorbic acid is not required for the mRNA expression of the collagen type I gene. However, AA induces collagen secretion and accumulation to form an ECM. These results are in accordance with the observations made by the groups of Franceschi (1992)[56] and Quarles (1992)[50]. They showed that untreated and AA-treated MC3T3-E1/C14 cells can express and secrete procollagens, but AA-deficient cells are not able to process procollagens to collagen fibrils. This may be explained by the essential role of AA in collagen biosynthesis. The collagen molecule contains the modified amino acids hydroxyproline and hydroxylysine, which are necessary for the assembly and the stability of the molecule. These special amino acids results from a postranslational modification involving the enzymes prolyl and lysyl hydroxalase. The hydroxylation reaction catalyzed by these enzyme requires oxygen, ferrous ions, α-ketoglutarate and ascorbic acid[82]. Ascorbic acid has been found to act as a compound necessary for the reduction of hydroxalase-bound ferric iron formed during

proline hydroxylation[83]. In other words, AA is required to keep the hydroxalase enzyme active, which is a necessary step for the proper processing of procollagen.

Treatment of MC3T3-E1/C14 cells with AA also induces the expression of an array of osteoblast-related genes, such as BSP, OPN, OC, and proteins like ALPase. Our results show that these genes are expressed at a very low, almost undetectable level in untreated (undifferentiated) cells. Their expression is greatly increased by the addition of AA to the cultures. This gene expression pattern is characteristic of osteoblast undergoing differentiation and have been observed by many groups (Quarles et al, 1992[50]; Franceschi et al, 1992[56]; Aubin, 1998[78]; Raouf et al, 2000[55]; Vary et al, 2000[54]). However, it is to be noted that there is no "absolute" gene expression pattern. In reality, marker genes are expressed at heterogeneous levels at different stages of differentiation, which have been well described and documented by Aubin (1998)[78]. The group of Franceschi (1992)[56] reported that in MC3T3-E1/C14 cells, OPN mRNA levels are comparable in untreated and AA-supplemented cells over time, whereas we have shown that in our cell culture system, OPN is expressed at a very low level by untreated cells. On another hand, similar to our results, Xiao and colleagues[84] showed that untreated MC3T3-E1/C14 cells express a very low, constant level of BSP mRNA over time, and that these levels are increased by the addition of AA to the cultures. It has been suggested that this observed *in vitro* heterogeneity of marker genes expression represent a real, comparable *in vivo* heterogeneity. Candeliere et al[85] showed that *in vivo*, preosteoblasts and mature osteoblasts respectively express different genes, depending on their localization in bone, reflecting the changes on the microenvironment and the environment where each cell is located. Thus, it is possible that the differential expression of marker genes in our MC3T3-E1/C14 cell culture system, compared to what other groups have reported, may be a consequence of small variations in the cellular microenvironment, perhaps including differences between techniques used.

When the cultures are further supplemented with β-glycerolphosphate, the ECM mineralizes progressively, starting at day 8 of culture over a 12-day period examined. The mineral deposited in MC3T3-E1/C14 cell cultures is rich in $Ca^{2+}$ and $P_i$ and has an X-ray diffraction pattern similar to hydroxyapatite, the mineral phase found in bone. The mineral preferentialy appears to accumulate towards the dish surface, where the matrix is

more mature. MC3T3-E1/C14 osteoblast cell differentiation, matrix formation, markers expression and mineralization was confirmed to be highly reproducible Thus, we conclude that our MC3T3-E1/C14 cell culture system is an excellent model to study how different factors might affect differentiation, maturation and mineralization of osteoblast cell cultures, and how transglutaminase enzymes might be involved in these processes.

## 5.2 Tissue transglutaminase is expressed at a constant basal level in undifferentiating, differentiating, and mineralizing MC3T3-E1/C14 cell cultures

The ultime goal of investigating tTG expression and activity in differentiating MC3T3-E1/C14 cells and in the ECM was to gain insight into the role of transglutaminases in osteoblast differentiation and ECM mineralization. Our findings show that the amount of tTG mRNA, tTG protein levels and its activity are constant in MC3T3-E1/C14 cells over the 12-day time period examined independent of how the cell cultures were treated. Despite the relatively low, constant levels of tTG protein detected by Western blotting and the similar low levels of tTG activity, tTG protein was neverthless well detected by immunohistochemistry in MC3T3-E1/C14 cells. In bone sections, tTG appears to be preferentially located to the osteoid and to osteoblasts as demonstrated by Kaartinen et al (2002)[28]. In contrast, in histologic sections of MC3T3-E1/C14 cell cultures, tTG expression appears ubiquitous throughout the cells and the matrix, with a pink staining intensity that does not vary with the treatment or the time in culture. Importantly, however, tTG localizes to the cytosol in untreated cells and accumulates in the ECM upon AA treatment, suggesting a role for tTG in matrix assembly. MC3T3-E1 cells elaborate a thick collagenous matrix throughout their differentiation. Another possible role of tTG in these differentiating cultures is matrix stabilization. Interestingly, the group of Verderio[86]demonstrated that extracellular tTG is located at the basal and apical surfaces of cells and at cell-cell contacts, where it colocalizes with latent forms of TGF-β, suggesting that tTG contributes to the binding of latent TGF-β to matrix proteins, such as fibronectin. Since active TGF-β enhances the binding of fibronectin to the cell surface and increases matrix formation (Akimov,

2001)[26], the expression of tTG at the cell surface is proposed to contribute to the stabilization of the matrix by controlling matrix storage of latent TGF-β. Thus, it could be interesting to investigate the effect of TGF-β on MC3T3-E1/C14 cell cultures. Tissue transglutaminase may also contribute to matrix stabilization through the polymerization of OPN. We have shown that mineralizing MC3T3-E1/C14 cell cultures contain polymerized OPN, which likely results from tTG crosslinking activity. Interestingly, Kaartinen and coworkers (1999)[47] have demonstrated that polymerized OPN increases the binding properties of OPN to collagen, which could contribute to the stabilization of the ECM.

Our data show that MC3T3-E1/C14 cells express and contain tTG protein and that it is active. tTG expression and activity is constant in osteoblasts, independent of the differentiation stage of the cells, suggesting that tTG likely plays a basic, fundamental role in osteoblast cells and their secreted matrix in general There is mounting evidence indicating the importance of tTG in cell adhesion. Overexpression of tTG has been shown to increase cell spreading (Gentile, 1992)[87]. Gaudry et al[88] showed that tTG is secreted in cells undergoing attachment and spreading, where it colocalizes with integrins at cell adhesion sites. It has been demonstrated that externalized tTG enhances fibronectin matrix formation via the interaction with integrins (Akimov, 2001)[26], therefore regulating cell adhesion. Furthermore, tTG has been proposed to act as an integrin co-receptor for fibronectin to promote cell adhesion (Akimov, 2000)[25]. Our results show a trend of higher tTG activity in the first day (Figure 27) of MC3T3-E1/C14 cultures and could represent a role in the attachment and spreading of the cells early in cell culture.

## 5.3 Factor XIIIa expression in MC3T3-E1/C14 cells

In this study, we have shown for the first time that the transglutaminase FXIIIa is expressed in MC3T3-E1/C14 osteoblasts. Although FXIIIa has been previously characterized in chondrocytes undergoing mineralization by Nurminskaya and colleagues (1998[43], 2002[39] and 2003[46]) and in articular chondrocytes by Rosenthal et al (2001)[40], our study is the first to clearly demonstrate FXIIIa in undifferentiated, differentiating and mineralizing MC3T3-E1/C14 osteoblast cell cultures. The presence of FXIIIa mRNA in MC3T3-E1/C14 cells at different time points and treatment of the cultures was demonstrated by RT-PCR. We observed a trend, however not statistically significant, of elevated mRNA expression relative to time in culture, especially in mineralizing cells (AA + βGP).

FXIIIa protein was also identified in MC3T3-E1/C14 cell extracts as two immunoreactive bands at 81 and 76 kDa. These data are supported by the pre-digestion of the samples with thrombin, after which the 81 kDa band disappears. Thus, we hypothesize that the 81 kDa band could represent the unactivated, intact FXIIIa, and the 76 kDa band would represent the activated form of FXIIIa, named FXIIIa', where the activating peptide has been released. It is interesting to note that in cell extracts, a fraction of FXIIIa appears already activated, since a band of 76kDa is observed on Western blots. This suggests that in MC3T3-E1/C14 cells, FXIIIa is either non-proteolytically activated by divalent ions or that it may be activated by the action of thrombin or other proteases such as calpain and cathepsin C.

The presence of an inactive form of FXIIIa in our cell extracts is confirmed by an *in vitro* transglutaminase activity assay, where samples pre-digested with thrombin show a significantly higher transglutaminase activity. However, this result was observed only when the cells were harvested with a buffer containing EDTA (Buffer B). In contrast, when the cells were harvested using a buffer without EDTA (Buffer A), we did not see any significant difference between thrombin pre-digested and undigested samples. Therefore, the presence of EDTA likely extracts more FXIIIa or enhances FXIIIa sensitivity to thrombin. The latter possibility is supported by Mary et al (1988)[33], who

demonstrated that in the absence of divalent ions, thrombin activates FXIIIa more rapidly, and becomes eventually degraded to lower molecular-mass peptides with a simultaneous loss of transglutaminase activity. Interestingly, the same investigators have also demonstrated that in the presence of divalent ions, FXIIIa becomes resistant to thrombin proteolysis. This result may seem contradictory, since transglutaminases are known to be $Ca^{2+}$-dependent enzymes. This is also true for FXIIIa, however, this isoform of transglutaminase is apparently very sensitive to divalent ions concentrations. It has been demonstrated that a concentration of 1-2 mM $Ca^{2+}$ is required for the enzymatic activity, but a concentration of 5 mM of $Ca^{2+}$ is enough to protect FXIIIa from proteolysis. This limited proteolysis in the presence of divalent ions has also been characterized for $Mn^{2+}$ and $Zn^{2+}$. When the $Ca^{2+}$ exceeds 50 mM, FXIIIa becomes active.

Interestingly, FXIIIa activity increases with time in culture. This result could suggest that at earlier time points (day 2), most of the FXIIIa has already been activated in the cell culture system, where the enzyme has probably an important, yet undefined, role. At later time points (days 6 and 12), the pool of inactive FXIIIa accumulates in osteoblasts. The presence of FXIIIa in MC3T3-E1/C14 cells was further characterized by immunohistochemistry, where we observed that FXIIIa expression is cytosolic in untreated cells and the protein is secreted to the matrix, like tTG, upon AA treatment. FXIIIa localization is also clearly polarized in the cells. This pattern of expression may represent a specific accumulation of FXIIIa at cell-cell junctions, which are numerous in more dense cultures (days 6 and 12). Interestingly, the localization of another isoform of transglutaminase, named TG1, has been reported to concentrate at adherent junctions[89] in a pattern similar to the one observed for FXIIIa.

In cultures supplemented with AA, the ECM shows a moderate staining, which increases at later time points (day 12). This result suggests that a portion of FXIIIa is externalized and becomes incorporated into the ECM, where its role remain to be defined. Abundant evidence demonstrates that FXIIIa is released, by an as yet unknown mechanism, to the extracellular matrix (Ueki et al, 1996[90], Adany et al, 2003[37]; Nurminskaya et al, 2002[39]; Nurminskaya et al, 2003[46]; Rosenthal et al, 2003[40]). Secreted FXIIIa could have a role in cell-matrix stabilization, as suggested by the study of Paye and Lapiere[91] who demonstrated that FXIII enhances the fibronectin-mediated adhesion

of cells to collagen type I. Interestingly, FXIIIa has also been proposed to be involved in the regulation of collagen synthesis[92]. Like tissue transglutaminase, FXIIIa has been reported to promote cell adhesion and cell spreading mediated by integrin interactions (Ueki et al, 1996)[90]. However, the amount of data concerning FXIIIa implication in cell adhesion is limited.

# 6. CONCLUSIONS

Noncollagenous proteins are prominent components of ECM and are implicated in cell-cell and cell-matrix communication, and in mineralization. Recent studies have suggested a role for the transglutaminase enzymes, especially the isoforms tTG and FXIIIa, in matrix stabilization and mineralization of skeletal tissues.

The goal of our work was to characterize transglutaminase expression and activity in an *in vitro* osteoblast cell culture model and to provide insight on its possible functions in bone matrix development. The results presented in this thesis show tTG expression, activity and localization in the preosteoblast MC3T3-E1/C14 cell culture model. Our data describe for the first time FXIIIa expression and activity in osteoblasts. The novel characterization of FXIIIa in osteoblast cultures, and the co-expression of FXIIIa and tTG in MC3T3-E1/C14 cells argue for a potentially redundant, fundamental role for transglutaminases related to their putative functions. The absence of clinically defined skeletal defects in tTG or FXIIIa-deficient mice support this notion[93-94].

Additional work is in progress to further characterize and localize FXIIIa and tTG in MC3T3-E1/C14 cells by fluorescence microscopy of whole cells. Enzyme inhibition studies will be carried to further investigate the functions of transglutaminases in differentiating osteoblast cultures.

# 7. FIGURES

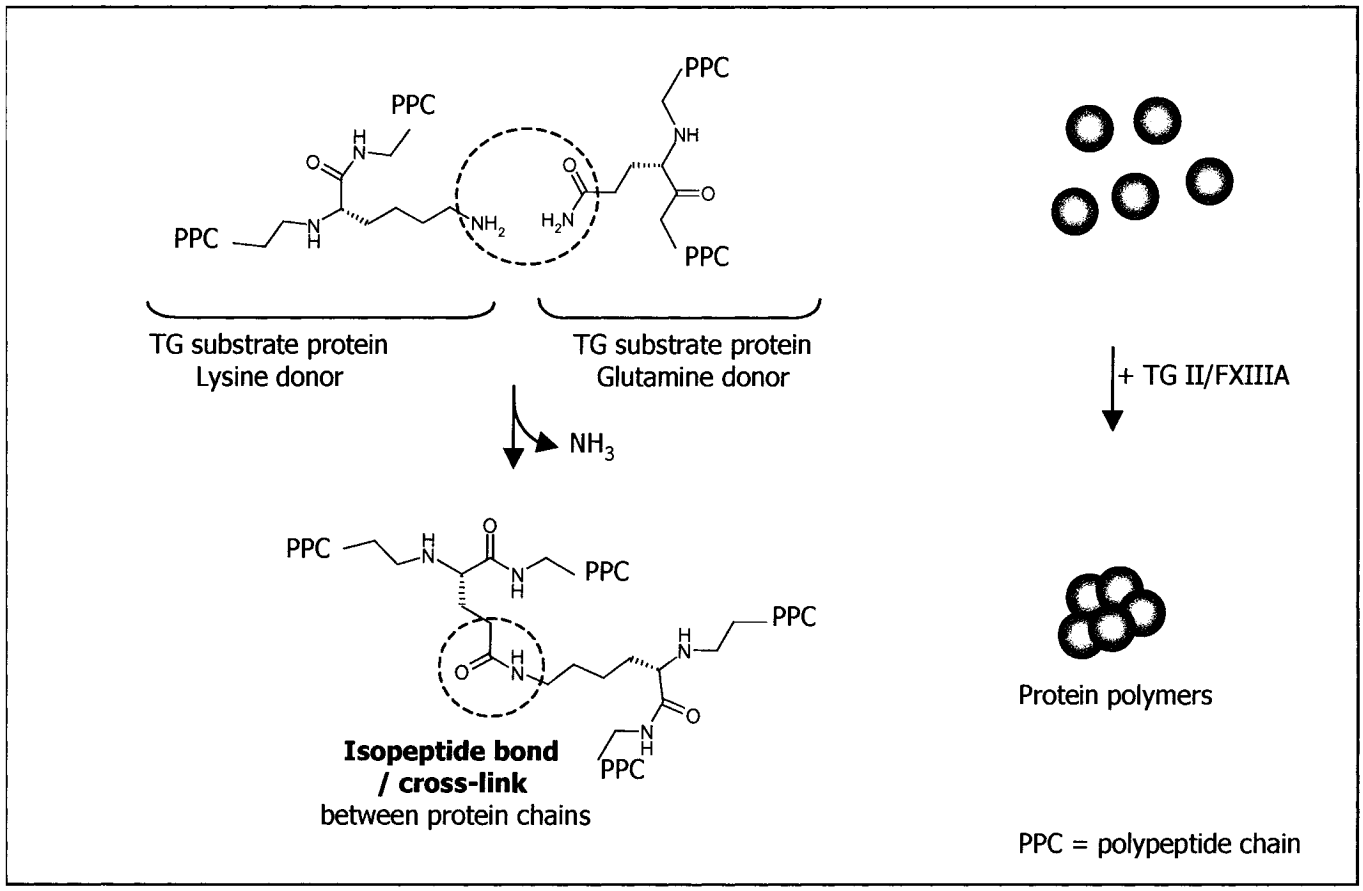



**Figure 1. Transglutaminase cross-linking reaction.** TGase catalyzes a transferase reaction between the γ-carboxamide group of a protein-bound glutamine and the ε-amino group of a protein-bound lysine residue or other various primary amine. The result of this posttranslational modification is a covalent, irreversible protein crosslink.

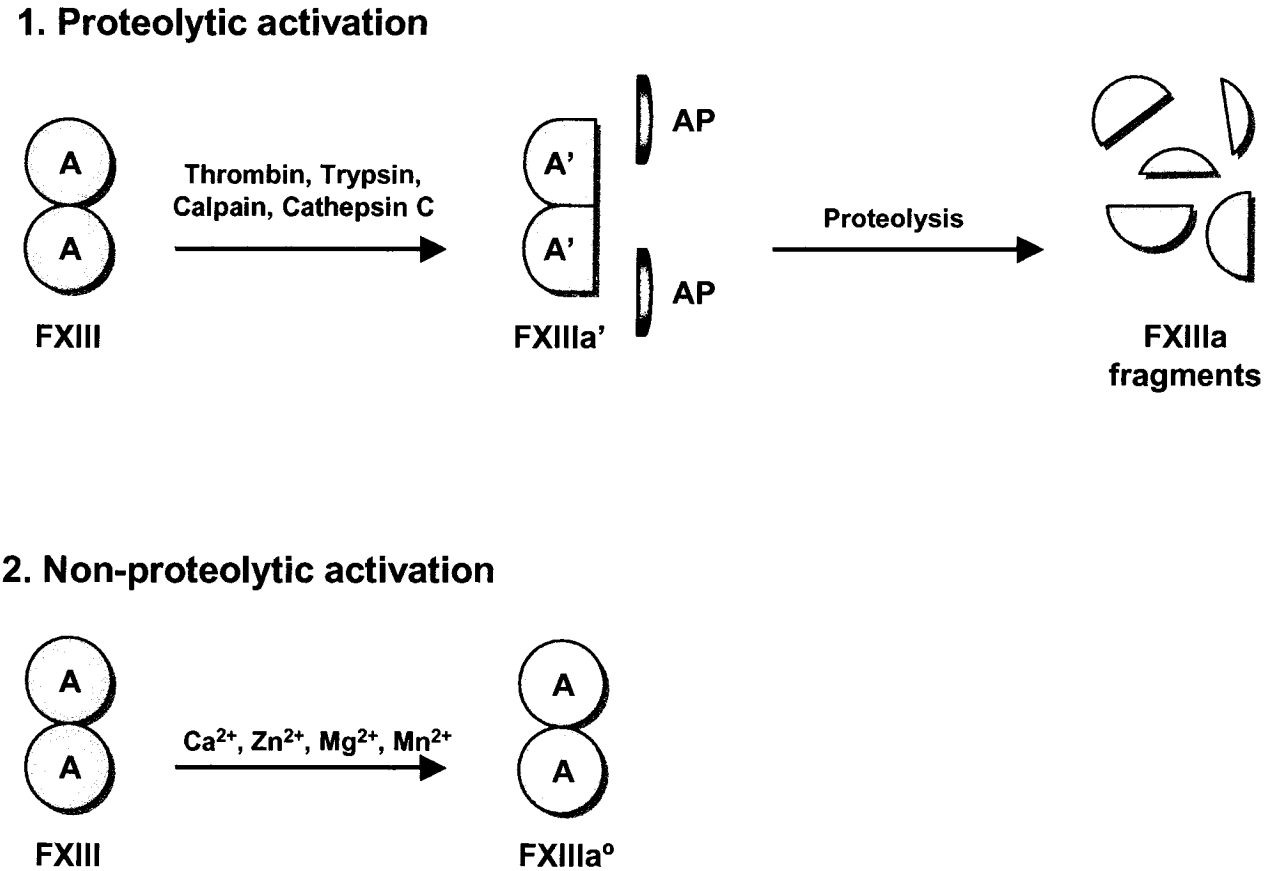



**Figure 2. Schema of FXIIIa activation.** Proteolysis by proteases such as thrombin, trypsin, calpain, or cathepsin C removes the activation peptide (AP) from inactive A subunits, which become activated (A') subunits. Activated A' subunits can be further cleaved by proteolysis, leading to FXIIIa fragments with reduced enzyme activity. Inactive A subunits of FXIII can assume an active A° configuration following a nonproteolytic activation by group IIA metal ions. Figure adapted from Muszbek L. et al (1996) [30].

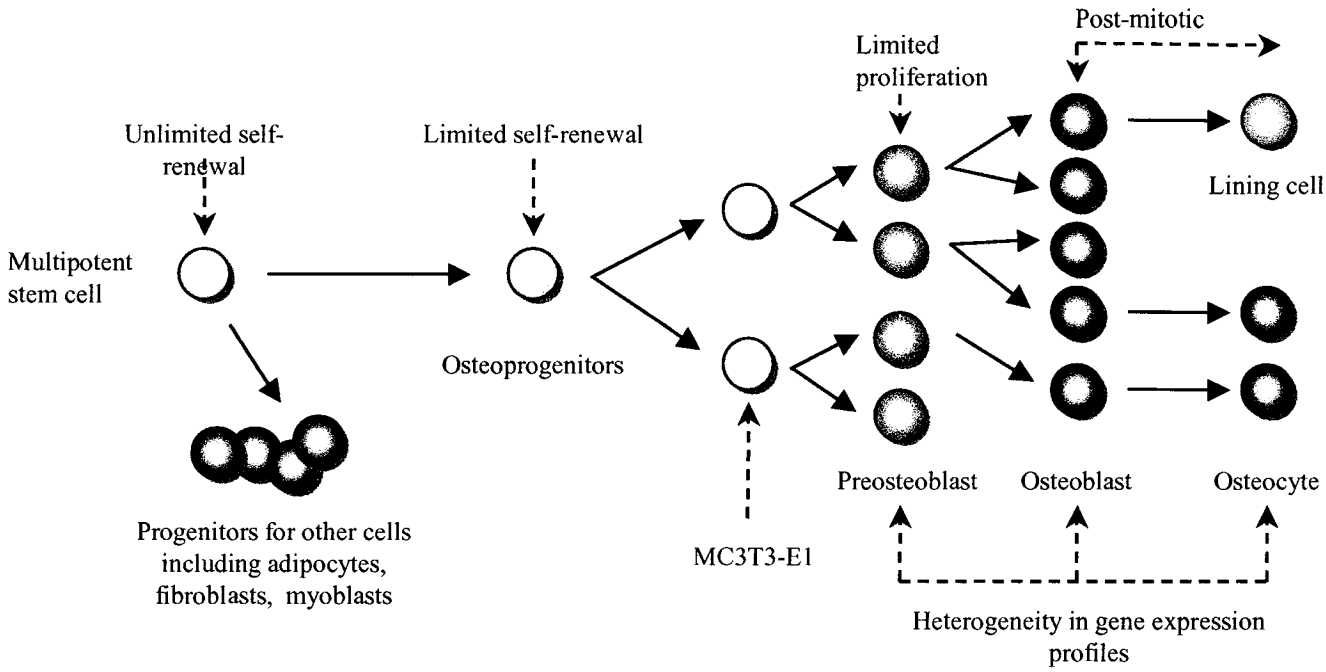



**Figure 3. Distinctive developmental stages of cells in the osteoblast lineage.** A mesenchymal multipotent stem cell is initially recruited to become an osteoprogenitor cell. Osteoprogenitors differentiate into osteoblasts. As the osteoblasts secrete bone matrix, they become trapped in their lacunae and are thereafter known as osteocytes. Each presumed stage of differentiation is characterized by a temporal pattern of gene expression. Figure adapted from Aubin (1998) [78].

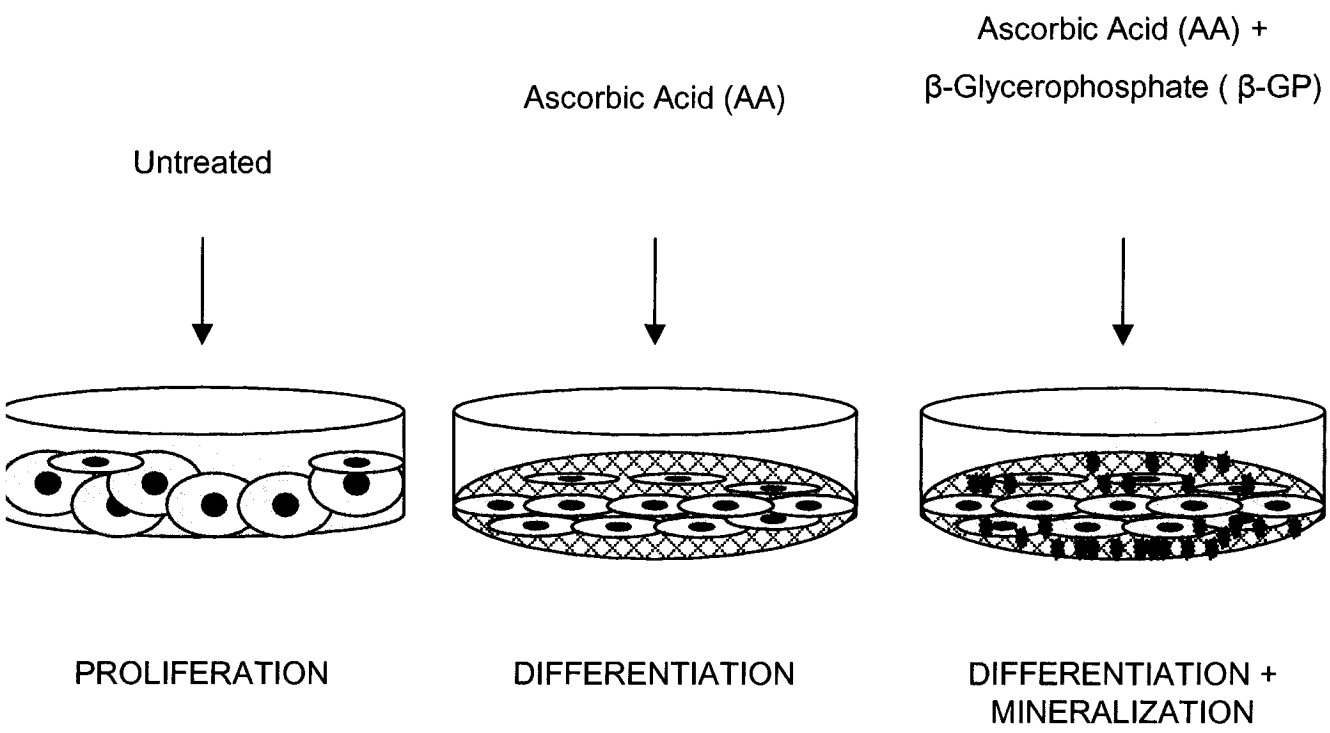



**Figure 4. Schema of the stages displayed over time by MC3T3-E1 cells after different treatments.** MC3T3-E1 preosteoblast cells proliferate with time when they are grown in complete α-MEM. Upon supplemention with AA, MC3T3-E1 cells differentiate to mature osteoblasts over time. After supplementation with AA and βGP, MC3T3-E1 cells differentiate with time into mature osteoblasts and the cell cultures mineralize.

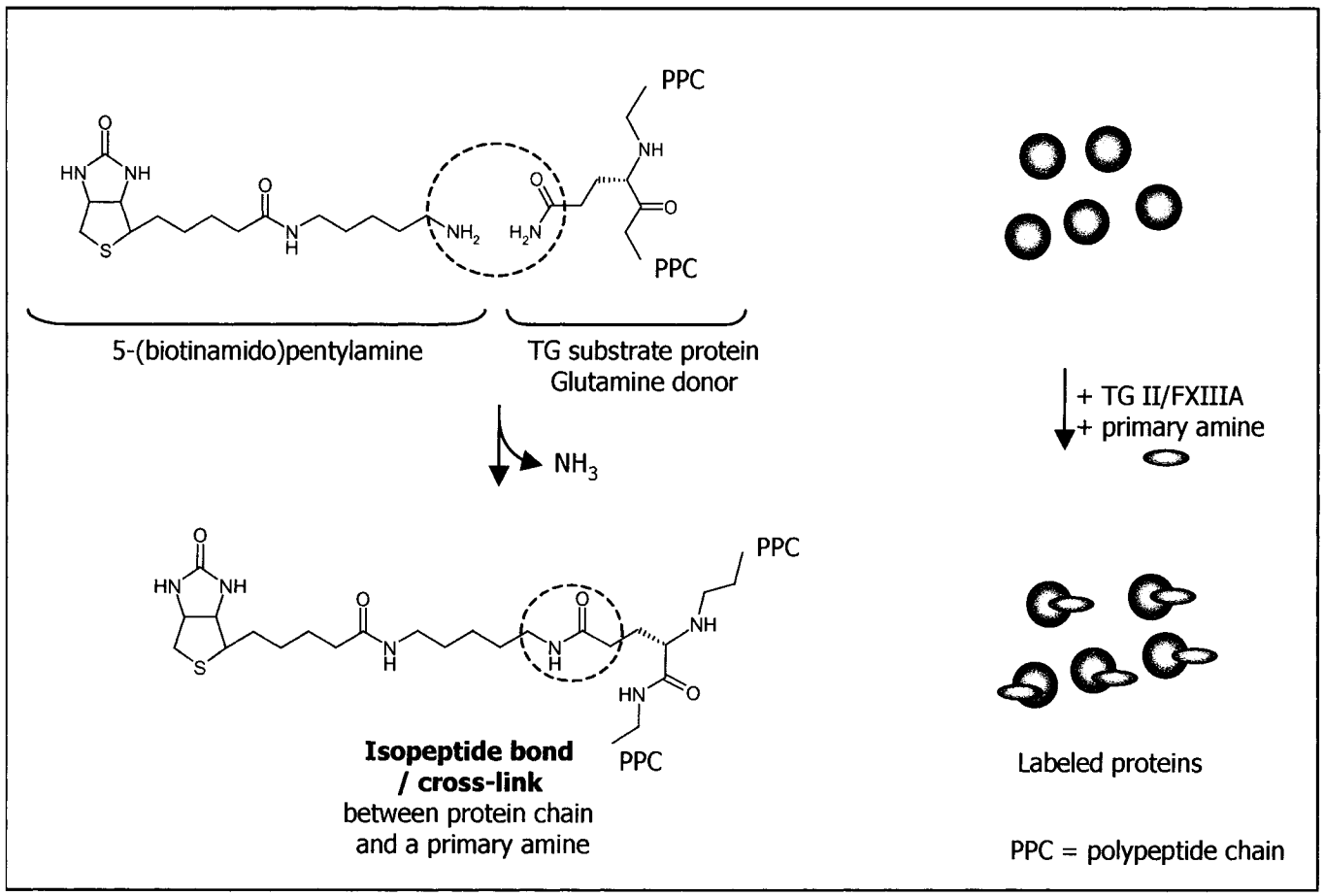



**Figure 5. Transglutaminase cross-linking and labeling reaction.** TGase catalyzes the ε-amino group of a protein-bound lysine residue or various primary amine. In the TGase activity assay, the reactive glutamine residue is provided by fibronectin and the reactive lysine is the substrate 5-(biotinamido) pentylamine. The product of this reaction is therefore labeled with biotin and detectable with an extravidin-conjugated antibody.

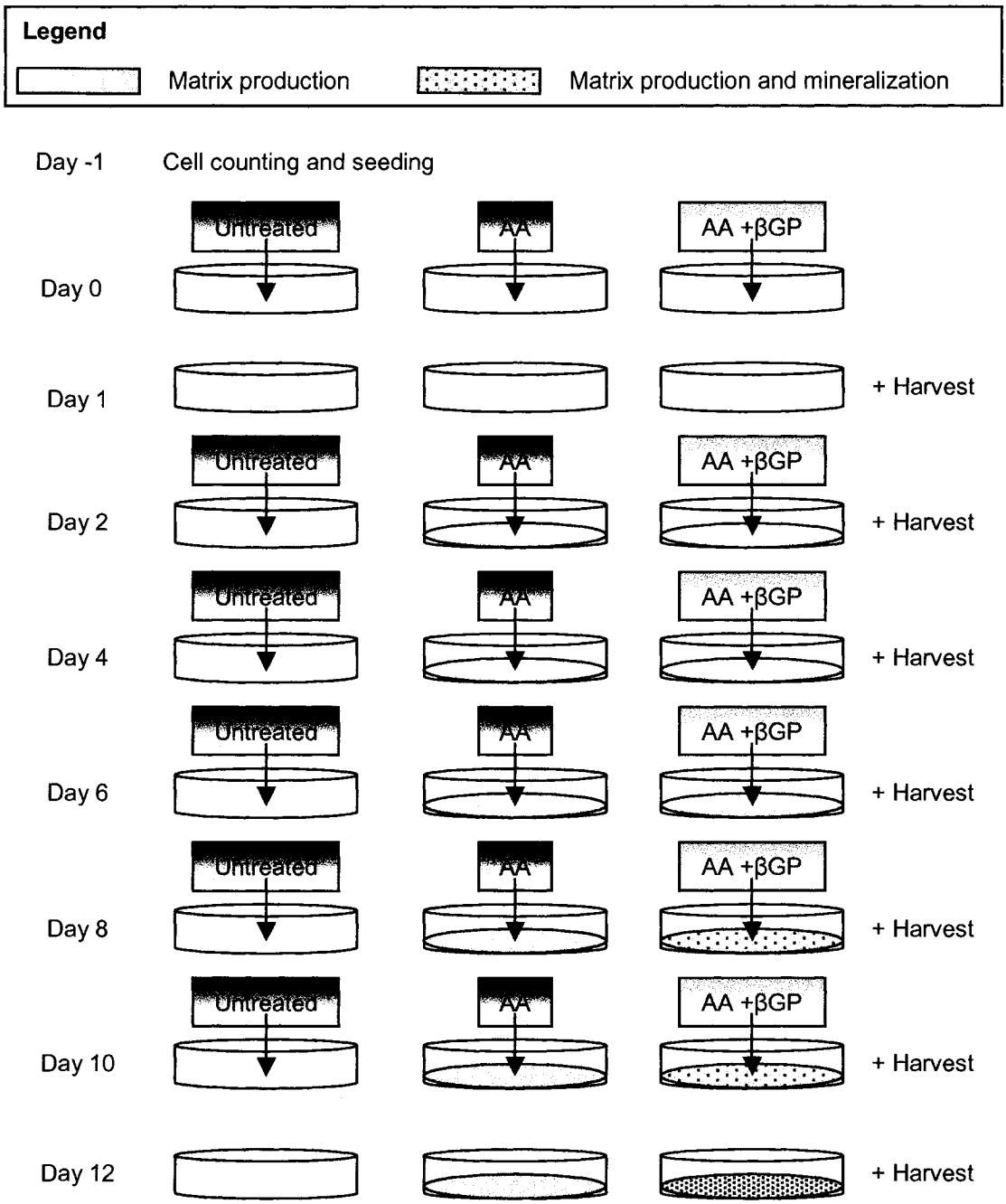



**Figure 6. Schema of different treatments on MC3T3-E1 cells.** At day -1, cells are counted, seeded onto culture dishes (35, 60 or 100 mm) and allowed to adhere for 24 h. At day 0, the media is changed and supplemented with AA or AA and βGP as per the experimental conditions tested. The media is changed every second day (days 2,4,6,8 and 10) . AA or AA and βGP are freshly added to the media each time. At days 1, 2, 4, 6, 8, 10 and 12, samples from each experimental conditions are harvested and submitted to different analyses.

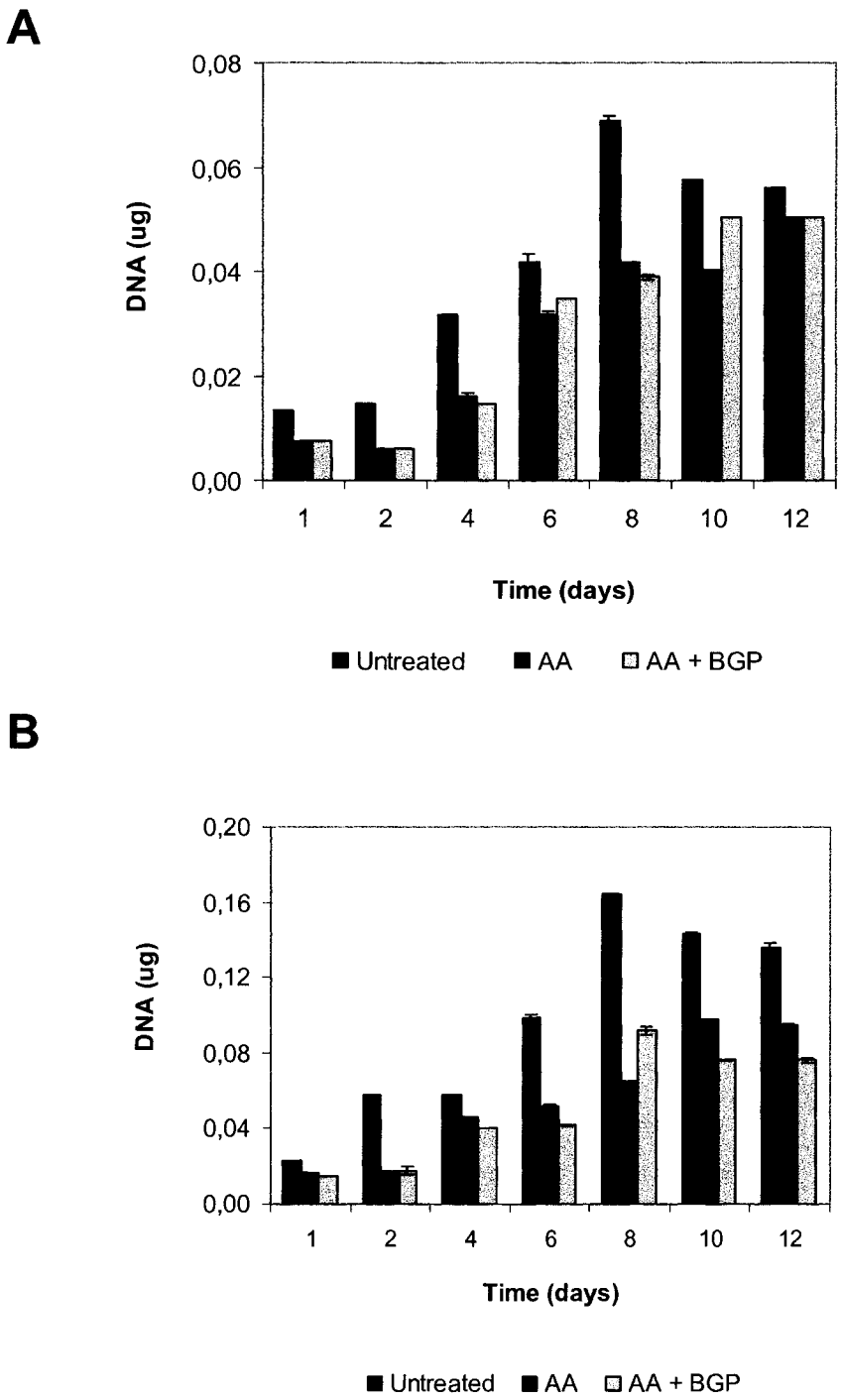
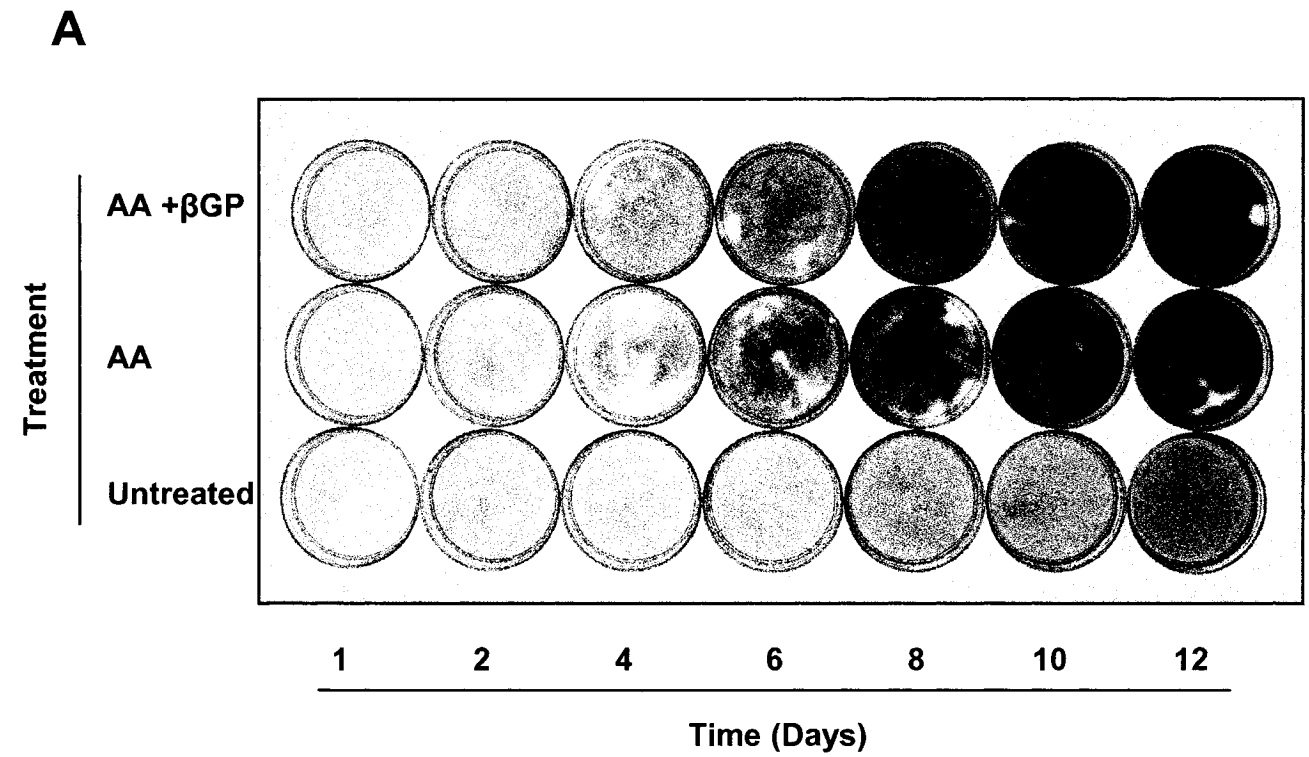



**Figure 7. DNA synthesis of MC3T3-E1/C14 cells relative to days in culture.** Cells were grown in **A)** 35 mm dishes or **B)** 60 mm dishes in the presence or absence of AA and βGP. At the indicated time points, cells were harvested and DNA was isolated and quantified by diphenylamine assay.



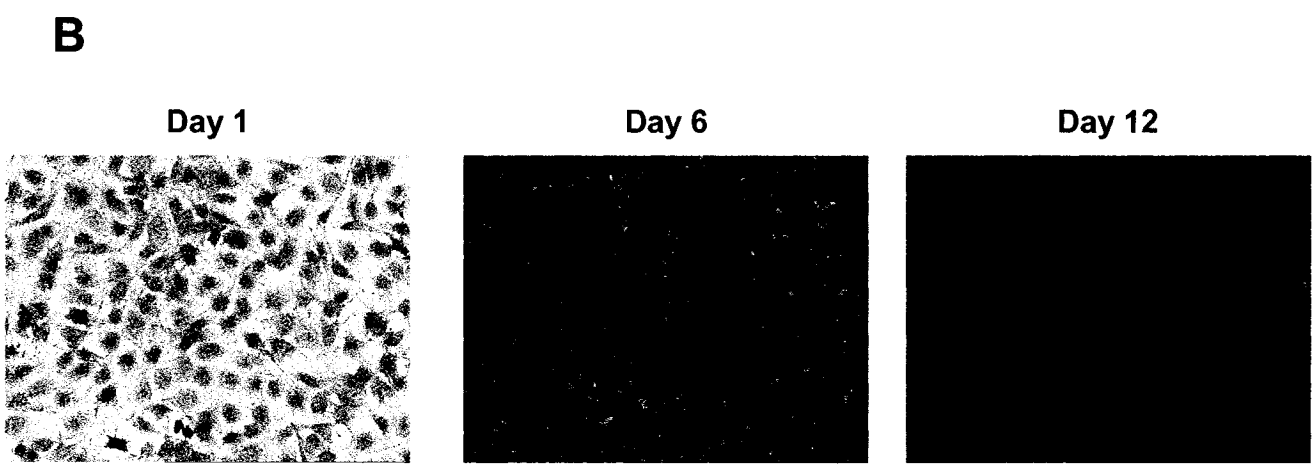




**Figure 8. Collagen production by MC3T3-E1/C14 cells visualized by sirius red staining.** **A)** MC3T3-E1/C14/ cells were grown with or without AA and βGP and stained with sirius red at the indicated time points. **B)** Higher magnification of MC3T3-E1/C14 cells treated with AA and stained with sirius red at days 1,6 and 12. Panel B magnification is x200.

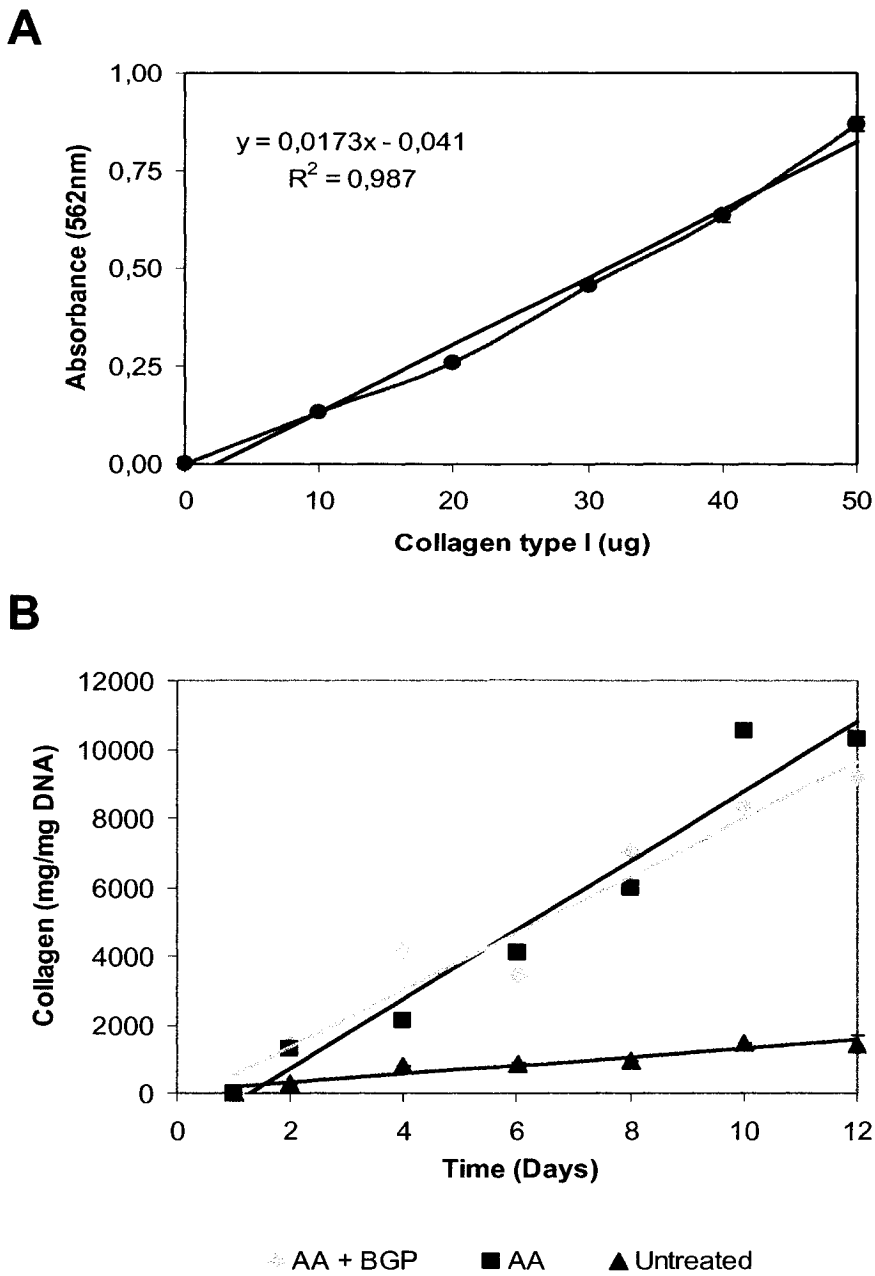
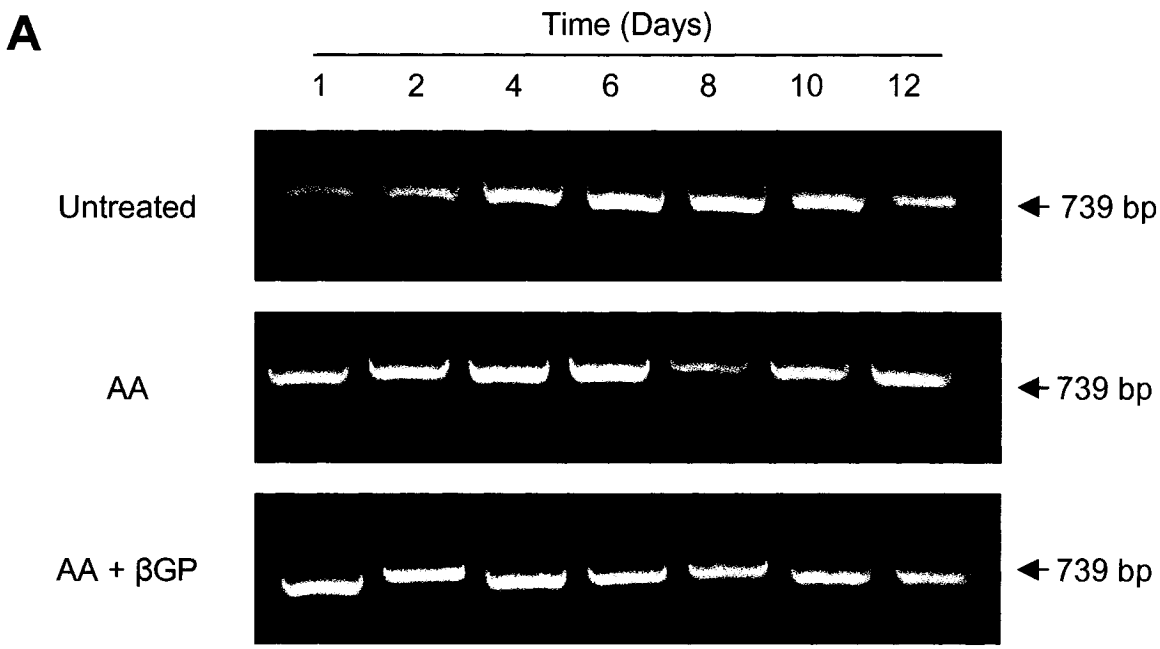



**Figure 9. Quantification of collagen synthesis by MC3T3-E1/C14 cells.** **A)** Standard curve made with different concentrations of soluble calf skin collagen type I and used for calculations. **B)** Cells were cultured in 35 mm dishes for the indicated times and incubated with, or without, AA and βGP. The cell layer was stained with sirius red (see Figure 5). The stained material was dissolved in 0.1 N sodium hydroxide, and the optical density was measured at 562 nm. All the values were normalized according to DNA content.



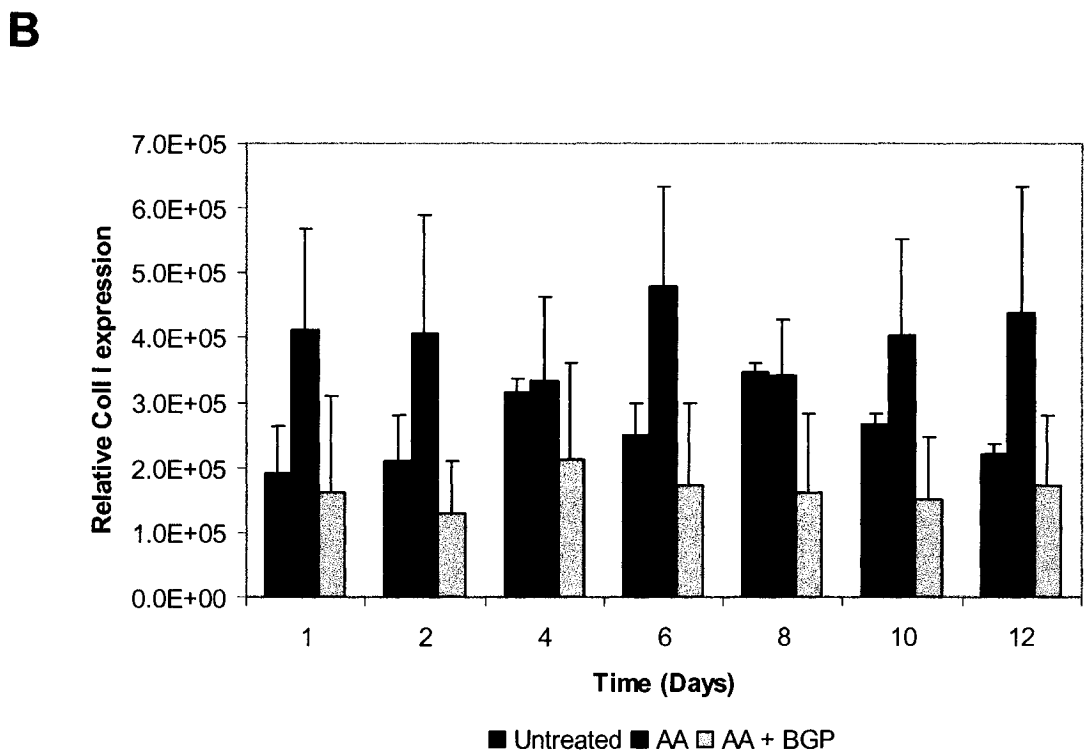




**Figure 10. Collagen type I (Coll I) mRNA expression in MC3T3-E1/C14 cells assessed by RT-PCR. A)** RNA prepared from cells cultured for the indicated periods with, or without, AA and βGP was analyzed by RT-PCR. **B)** Bands were quantified by volume analysis and GAPDH ratios were used to normalize values (Figure 11).

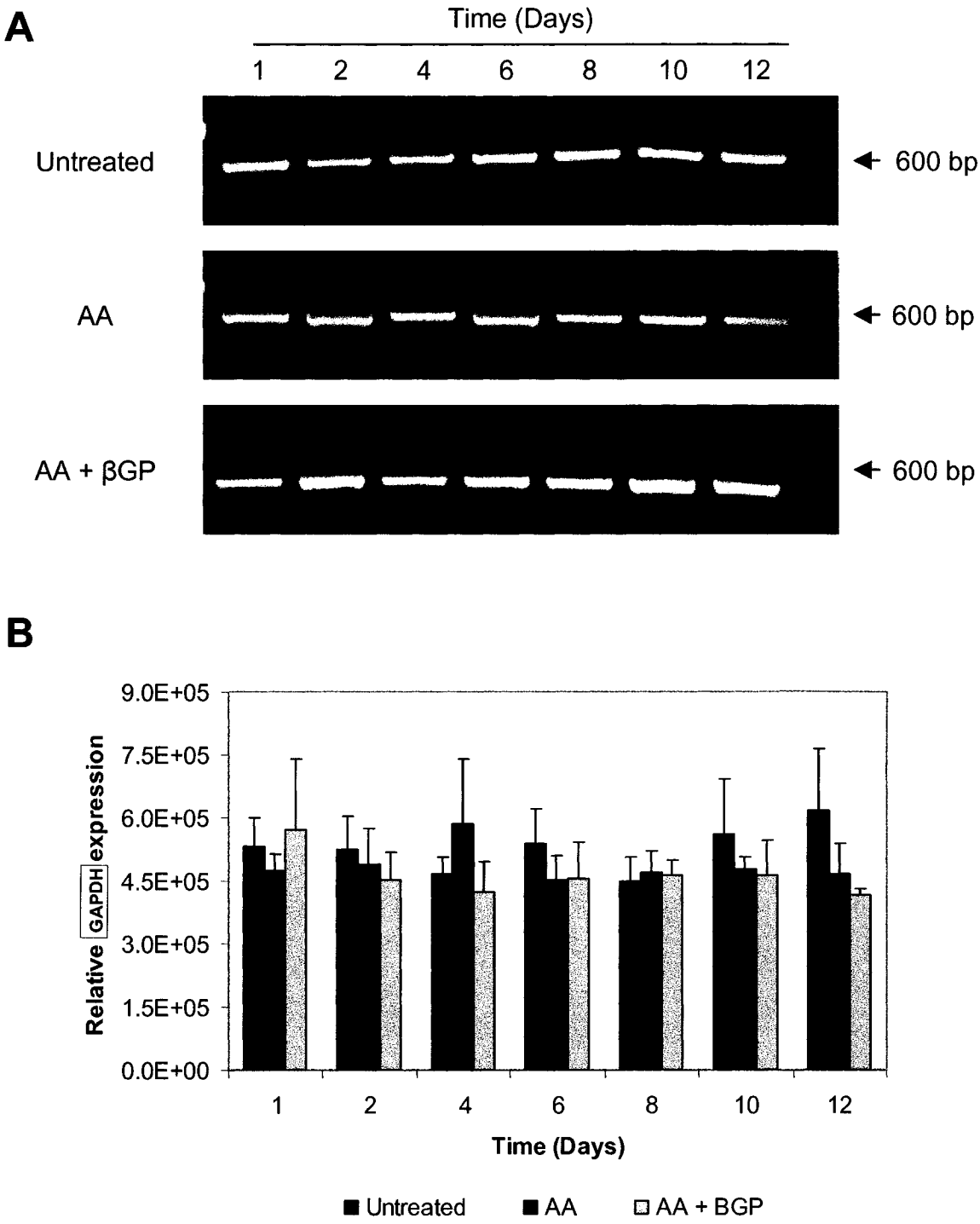
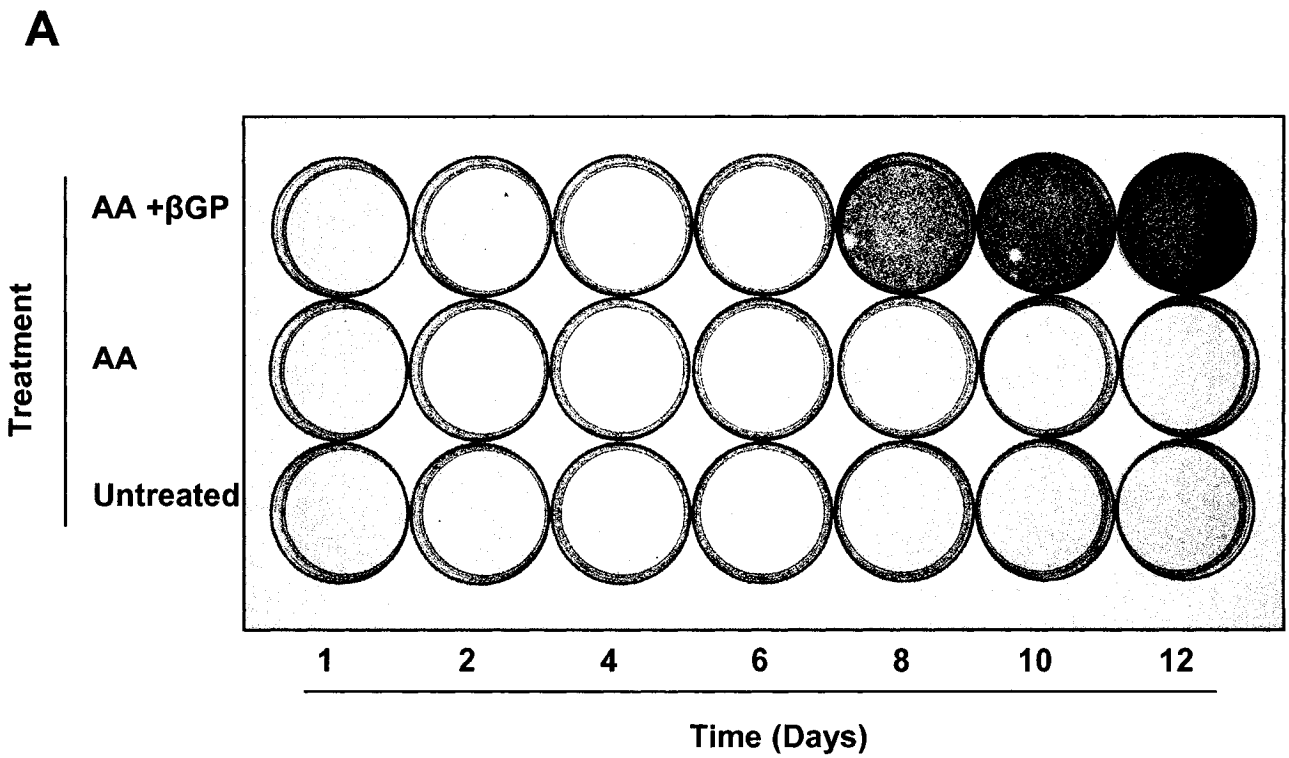



**Figure 11. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in MC3T3-E1/C14 cells assessed by RT-PCR. A)** RNA prepared from cells cultured for indicated periods with, or without, AA and βGP was analyzed by RT-PCR as a control. **B)** Bands were quantified by volume analysis and ratios were used to normalize other RT-PCR data.



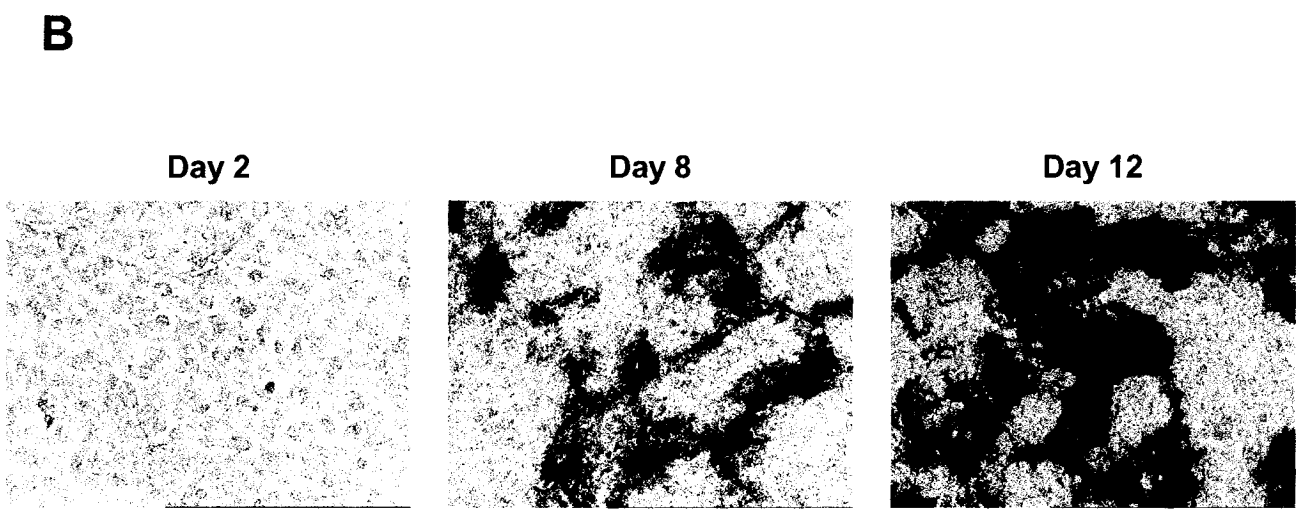




**Figure 12. Mineralization of MC3T3-E1/C14 cells assessed by von Kossa staining. A)** MC3T3-E1/C14 cells were cultured with, or without, AA and βGP and were stained with von Kossa reagent at the indicated time points. **B)** Higher magnification of MC3T3-E1/C14 cells subjected to AA and βGP treatment and stained with von Kossa reagent at days 2, 8 and 12. Panel B magnification is x200.

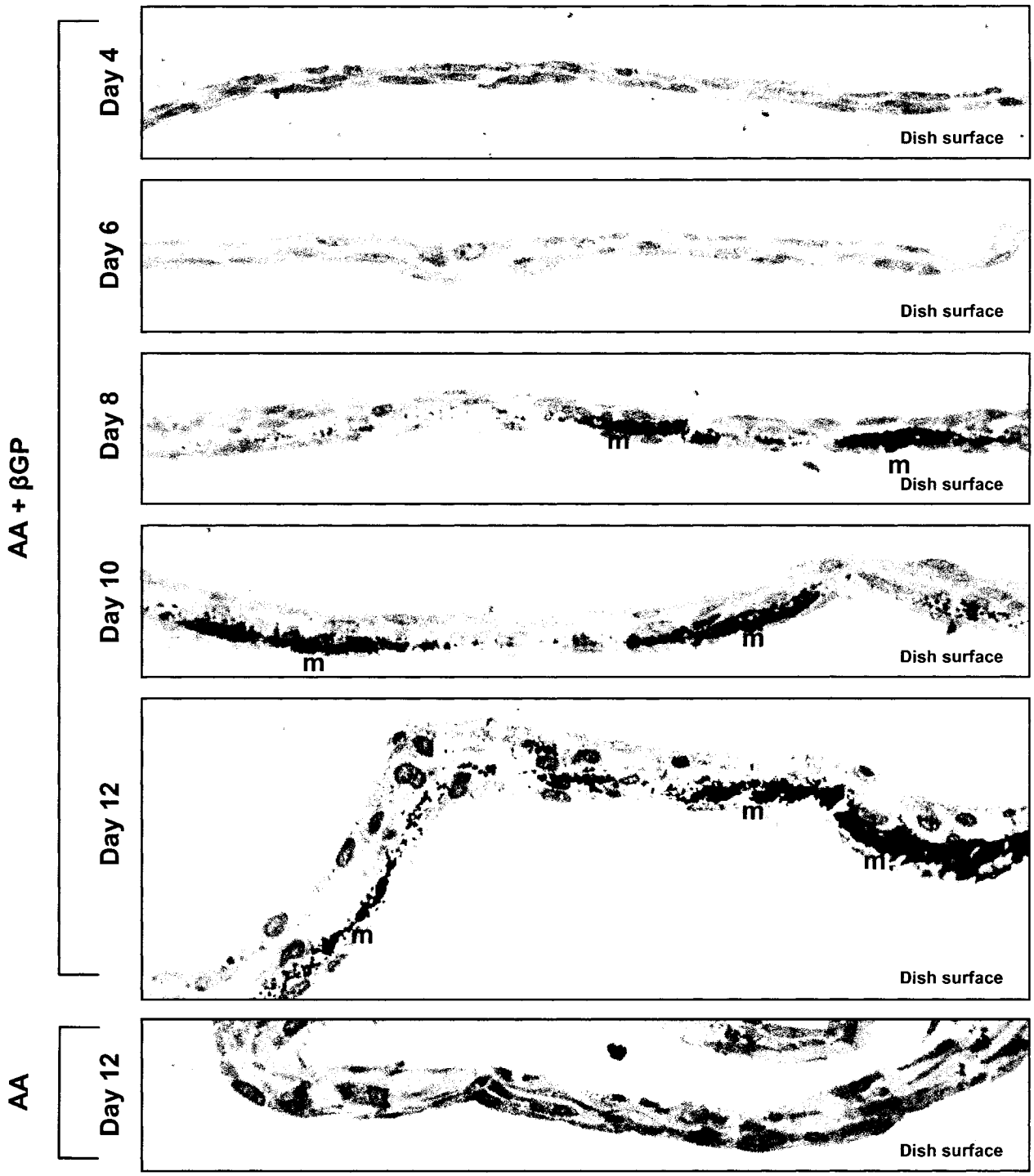



**Figure 13. Mineralization of MC3T3-E1/C14 cells visualized by von Kossa staining of plastic-embedded cells.** Cells were grown with the additions of AA and βGP, or with AA alone. At the indicated time points, cells were fixed and processed for plastic embedding. Sections were cut, stained with von Kossa reagent and counterstained with toluidine blue. Mineral deposition (m, black patches) starts at day 8 near the dish surface. Magnification is x200.

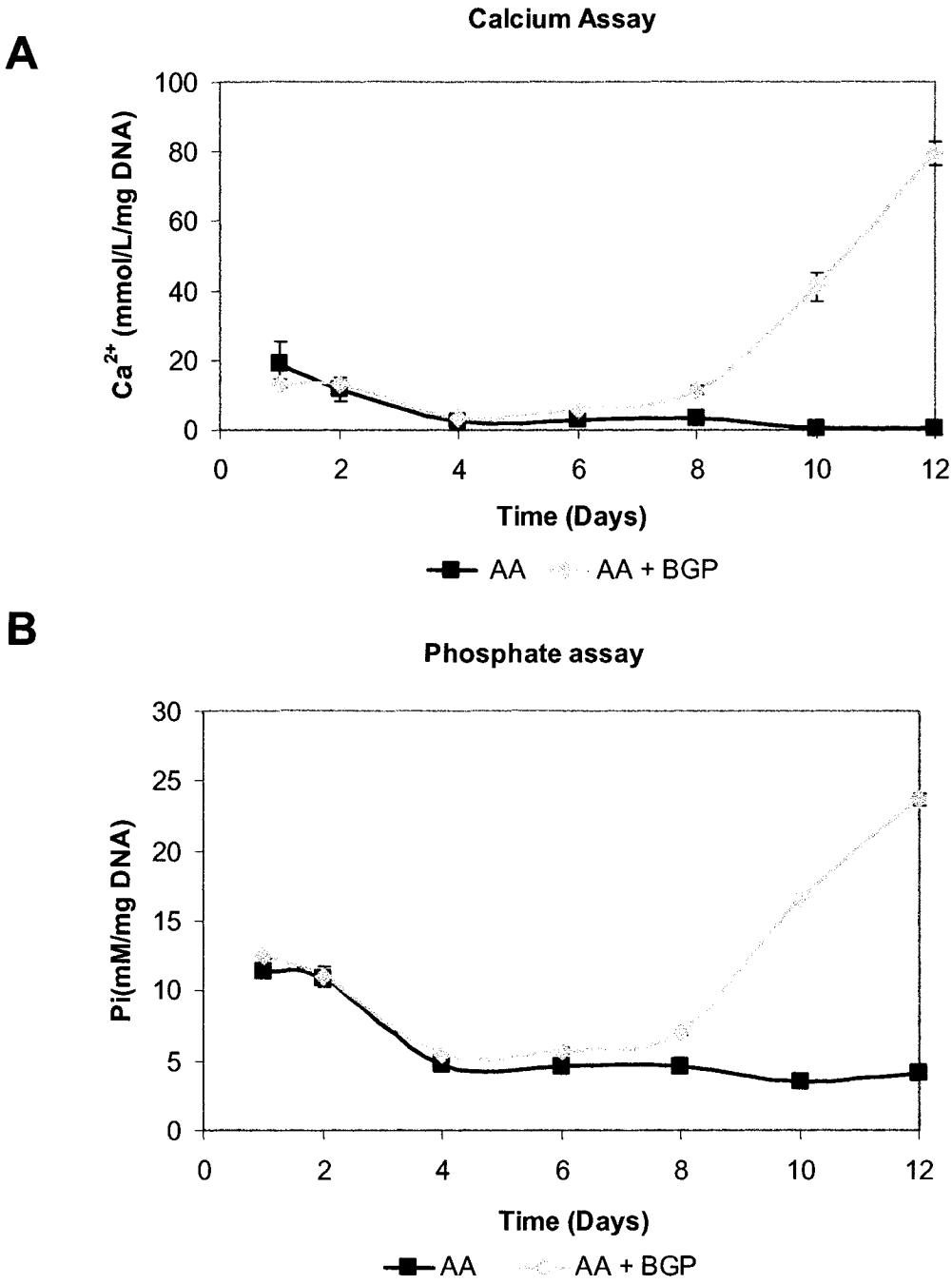



**Figure 14. Biochemical measurement of cell layer-associated calcium and phosphate.** Cells were plated on 60 mm dishes and cultures with, or without, AA and βGP. Samples were harvested at the indicated time points and analyzed for: **A)** cell layer-associated calcium; **B)** cell layer-associated phosphate. Data were normalized according to DNA content.

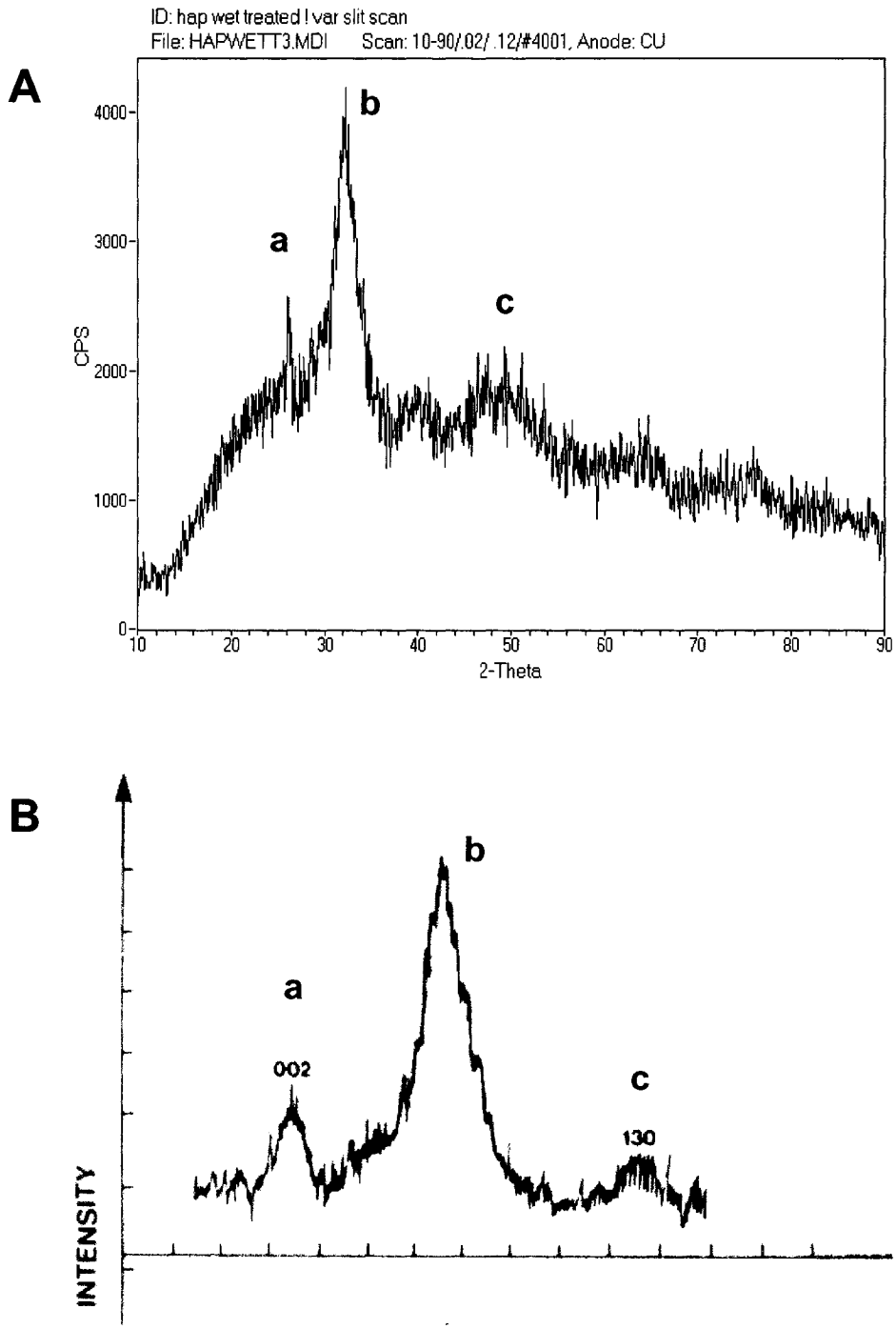



**Figure 15. X-ray diffraction pattern of hydroxyapatite. A)** Diffraction pattern of the mineral crystals deposited into the ECM of mature MC3T3-E1/C14 cells. **B)** Diffraction pattern of bone hydroxyapatite as published previously by Grynpas (1990)[95]. Matching spectral peaks (a, b, c) are typical for hydroxyapatite.

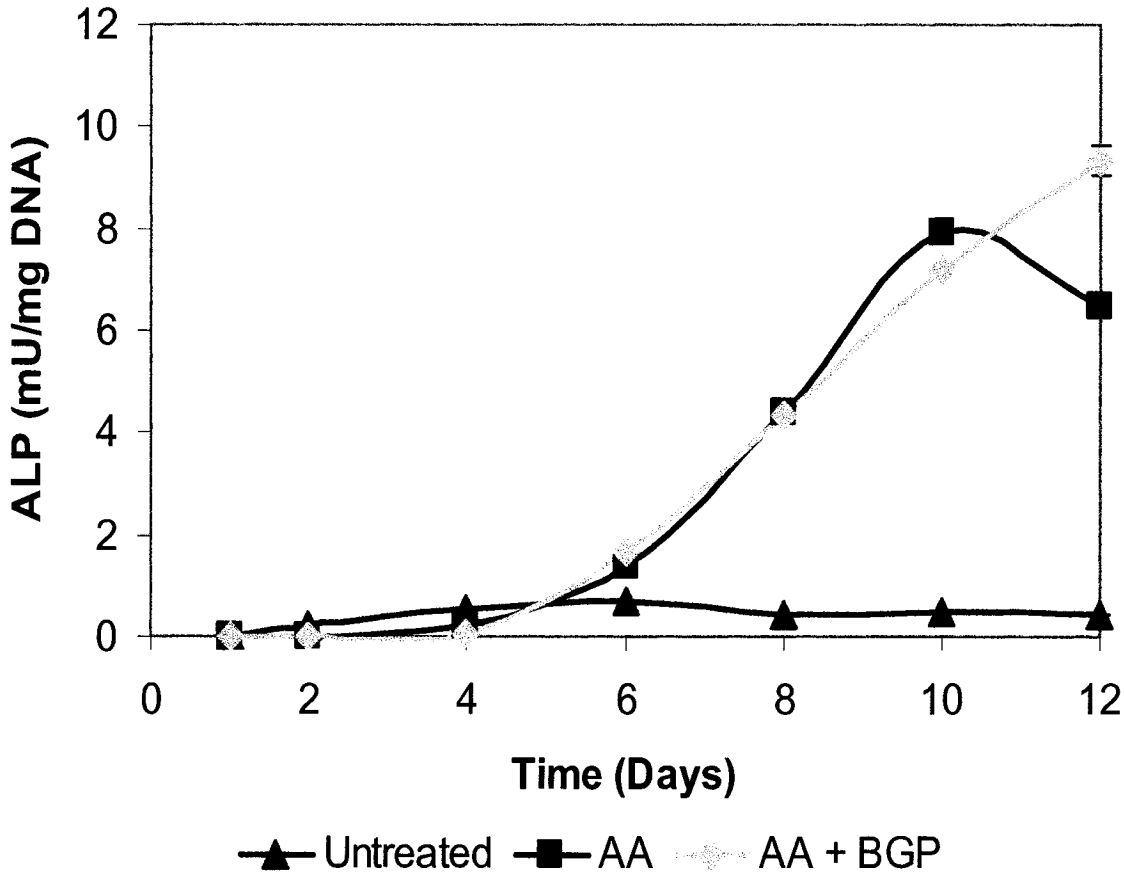
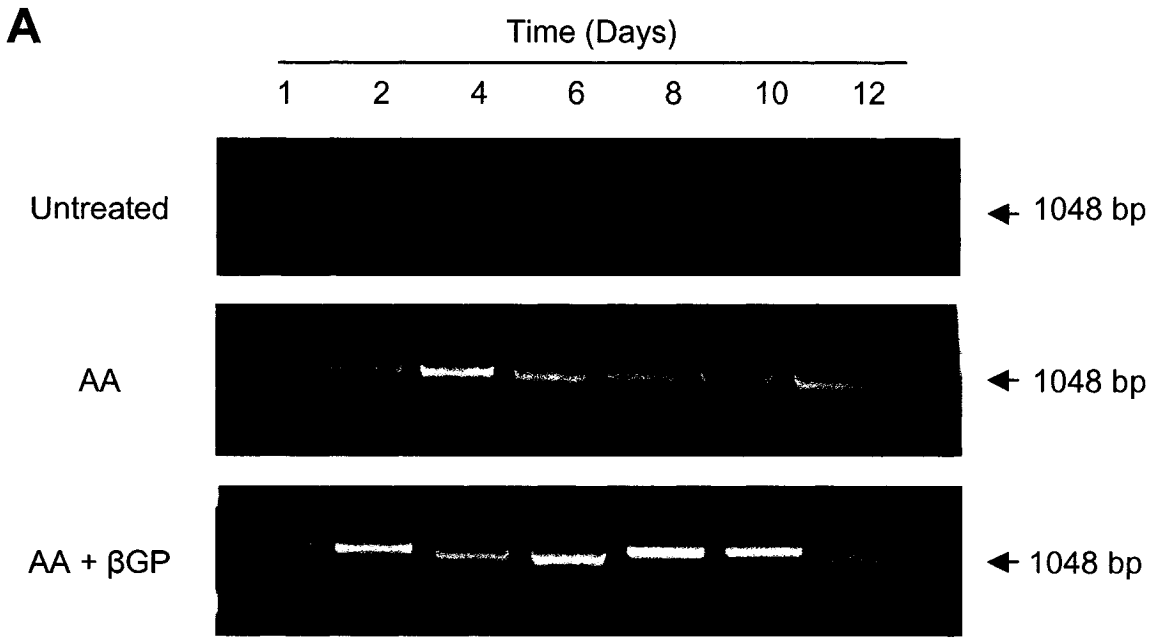



**Figure 16. Alkaline phosphatase (ALP) activity in MC3T3-E1/C14 cells during differentiation.** Cells were cultured in 60 mm dishes and treated with, or without, AA and βGP. At the indicated time points, cells were harvested and assessed for alkaline phosphatase activity. Data were normalized according to DNA content.



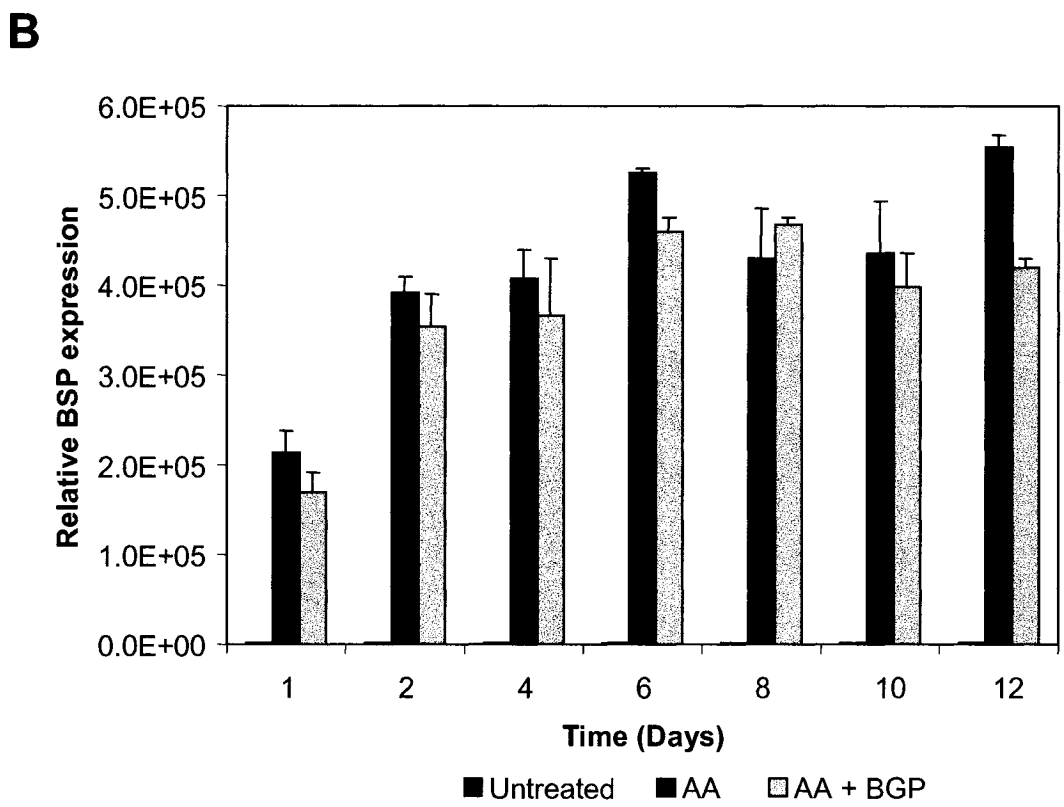




**Figure 17. Bone sialoprotein (BSP) mRNA expression in MC3T3-E1/C14 cells assessed by RT-PCR. A)** RNA prepared from cells cultured for the indicated periods with, or without, AA and βGP was analyzed by RT-PCR. **B)** Bands were quantified by volume analysis, and GAPDH ratios were used to normalize values.

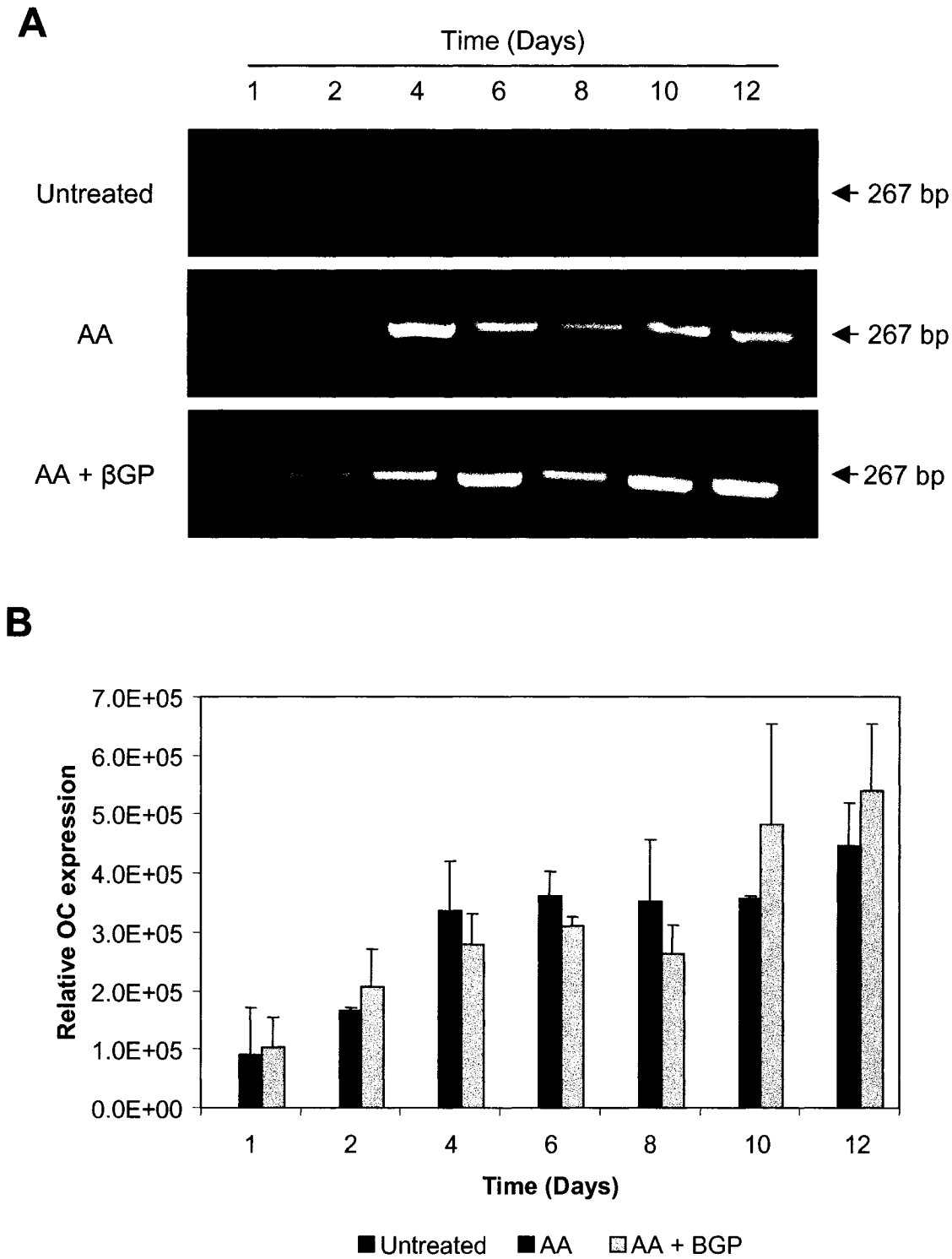



**Figure 18. Osteocalcin (OC) mRNA expression in MC3T3-E1/C14 cells assessed by RT-PCR. A)** RNA prepared from cells cultured for the indicated time with, or without, AA and βGP was analyzed by RT-PCR. **B)** Bands were quantified by volume analysis, and GAPDH ratios were used to normalize values.

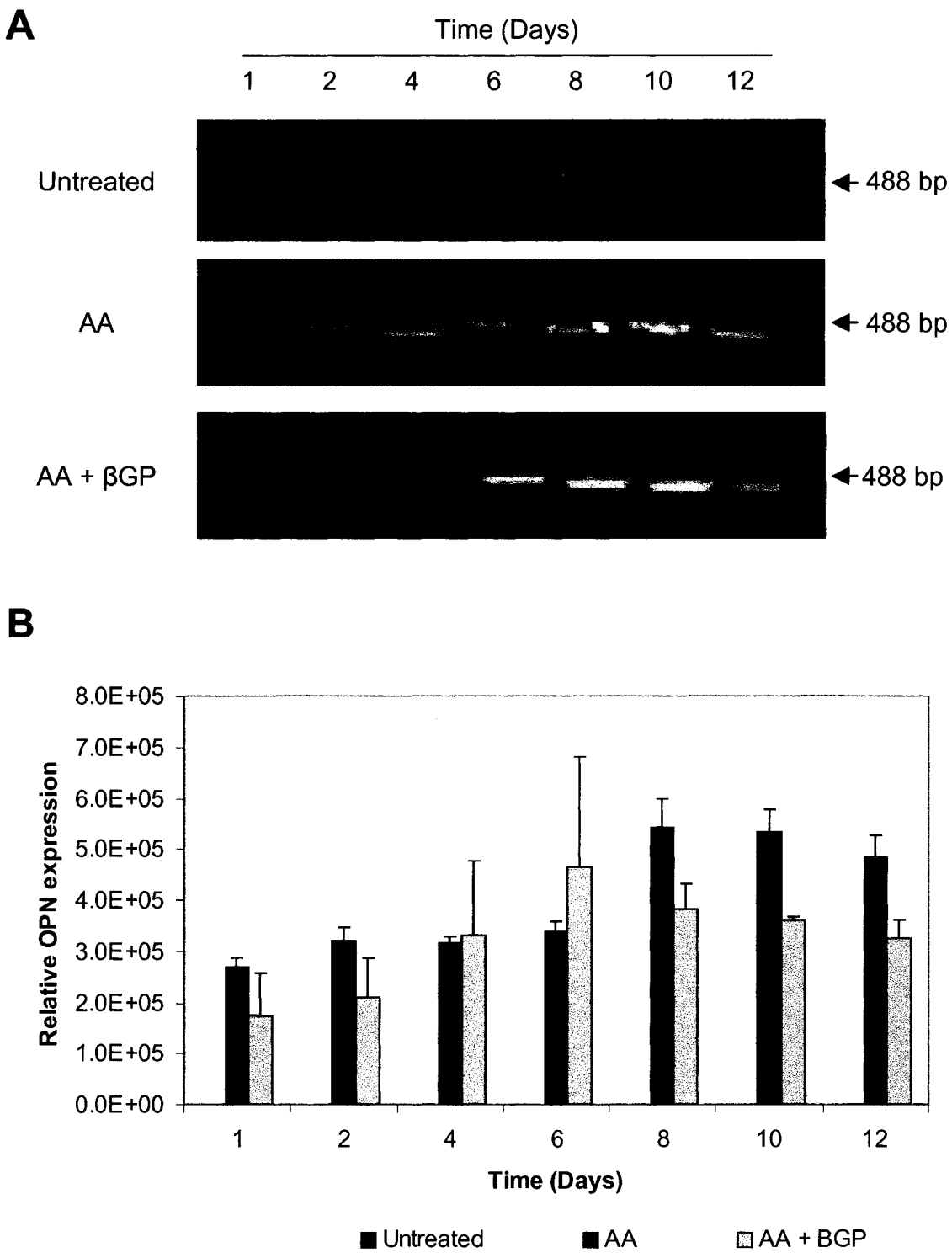



**Figure 19. Osteopontin (OPN) mRNA expression in MC3T3-E1/C14 cells assessed by RT-PCR. A)** RNA prepared from cells cultured for indicated periods with, or without, AA and βGP was analyzed by RT-PCR. **B)** Bands were quantified by volume analysis, and GAPDH ratios were used to normalize values.

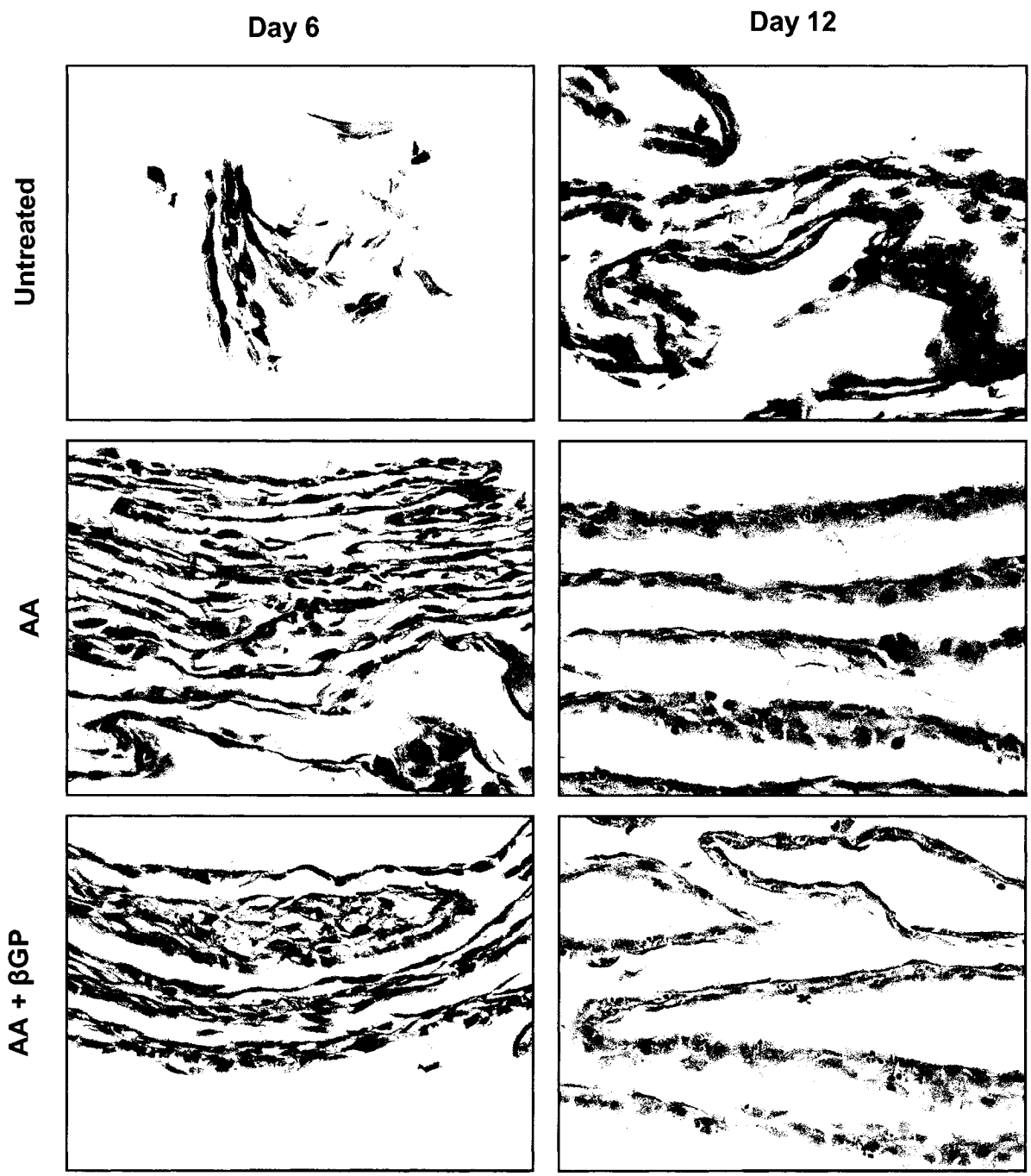



**Figure 20. Hematoxylin and eosin staining of paraffin sections of MC3T3-E1/C14 cells to show cell morphology.** Paraffin sections of MC3T3-E1/C14 cells that were grown for 6 and 12 days and treated with media only, AA, or AA and βGP. Magnification is x200.

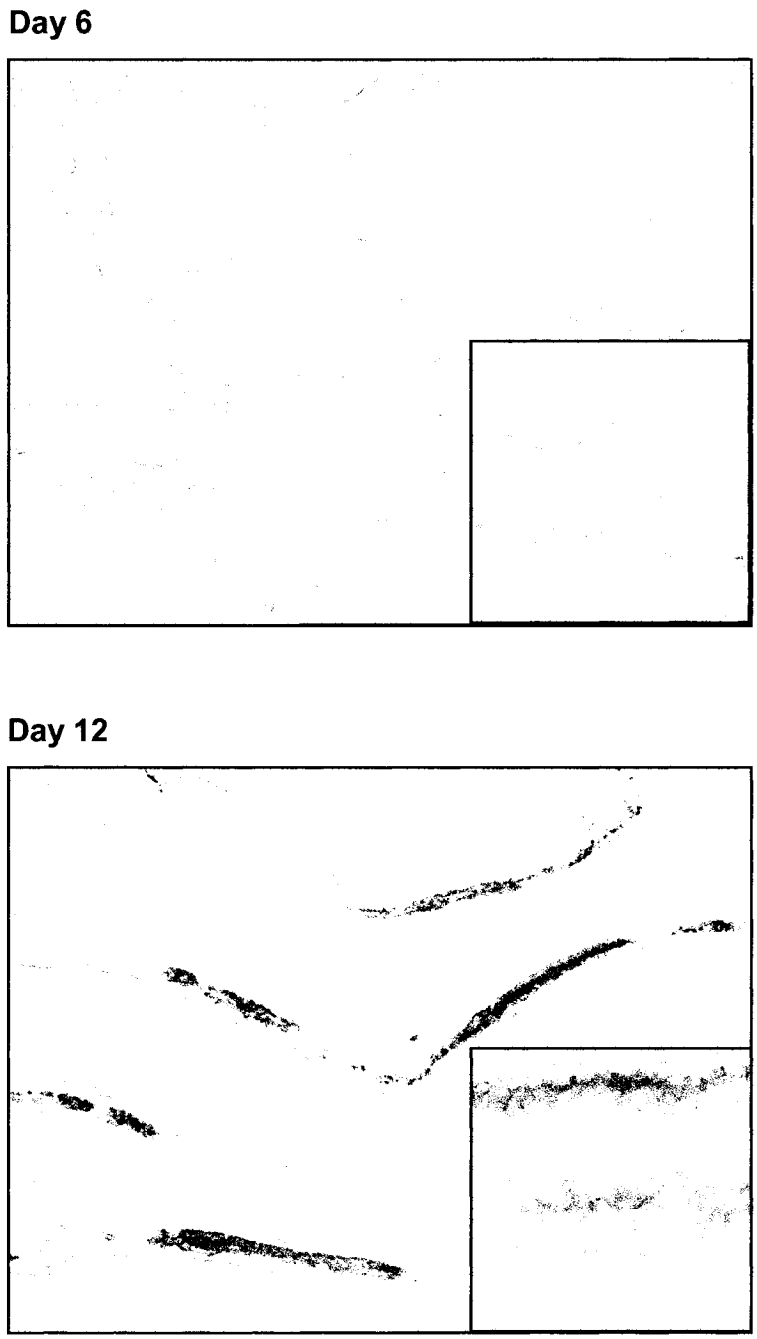



**Figure 21. Immunohistochemical localization of OPN in MC3T3-E1/C14 cells.** Paraffin sections of MC3T3-E1/C14 cells that were grown for 6 and 12 days and treated with AA and βGP. Immunostaining for OPN reveals a strong immunoreaction at the sites of mineralization in day 12 cultures,but not in day 6 cultures. Sections were counterstained with methyl green. Negative immunohistochemical staining control is presented in Figure 37. Magnification is x200 (insets x400).

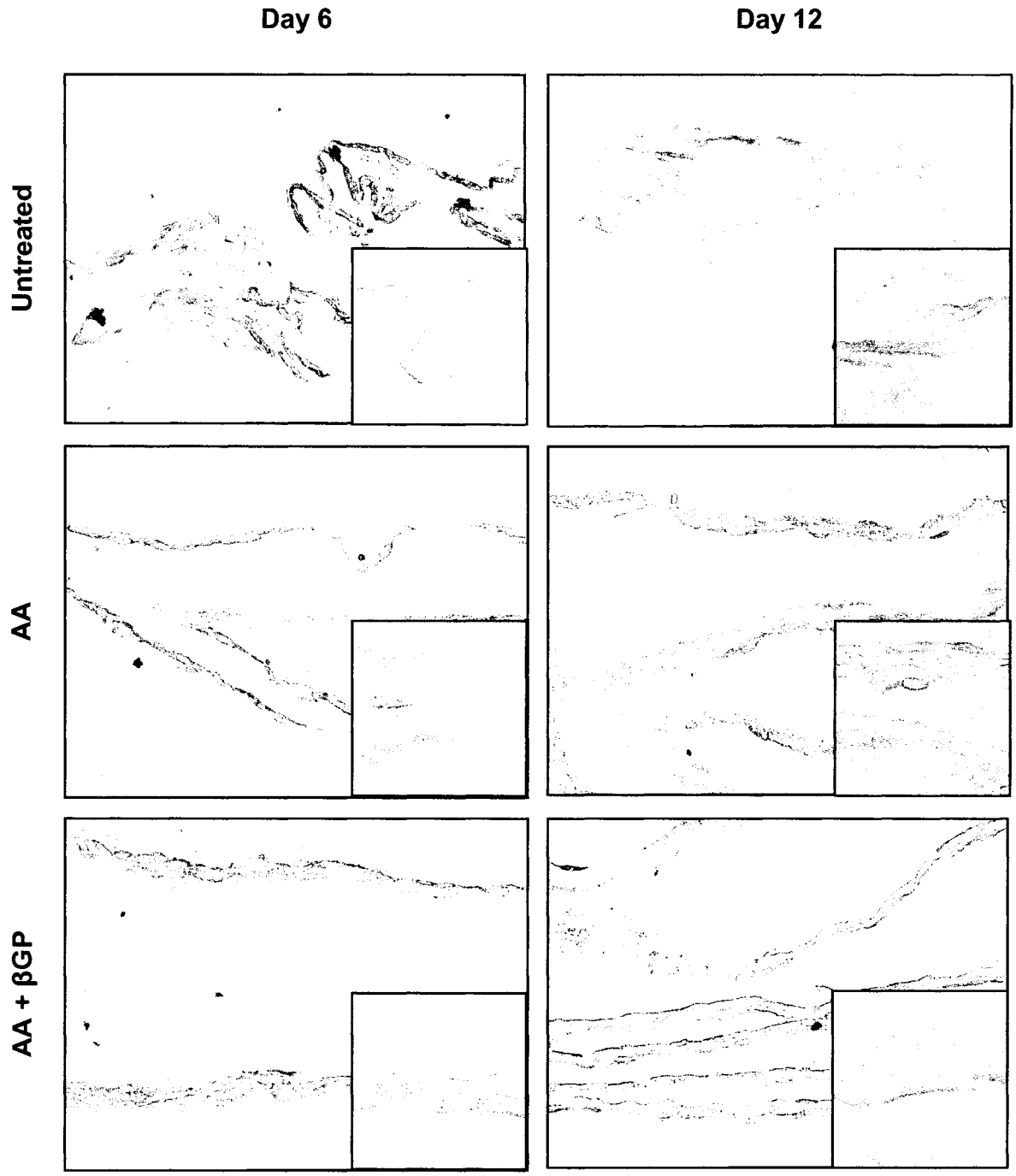



**Figure 22. Immunohistochemical localization of isopeptide bonds in MC3T3-E1/C14 cells**. Paraffin sections of MC3T3-E1/C14 cells that were grown for 6 and 12 days and treated with AA, AA and βGP, or untreated. Immunostaining for isopeptide bonds reveals a general staining of the ECM and the cells, independent of experimental conditions tested. Sections were counterstained with methyl green. Negative immunohistochemical staining control is presented in Figure 37. Magnification is x200 (insets x400).

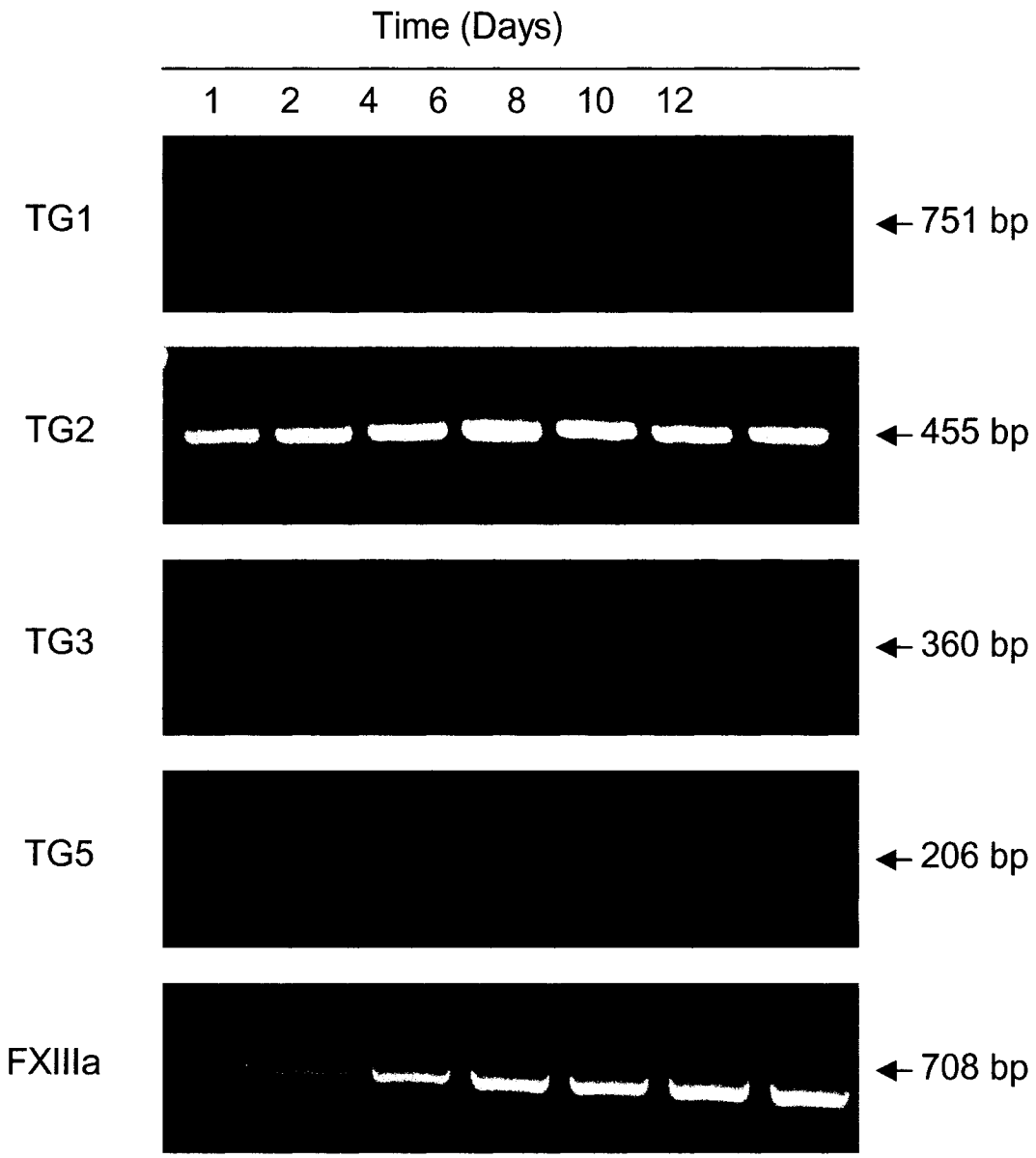



**Figure 23. Transglutaminase isoforms mRNA expression in mineralizing (AA + βGP treated) MC3T3-E1/C14 cells assessed by RT-PCR.** RNA prepared from cells cultured for the indicated periods with AA and βGP was analyzed by RT-PCR. TG1 = Keratinocyte transglutaminase; TG2 = Tissue transglutaminase; TG3 = Epidermal transglutaminase; TG5 = Transglutaminase 5; FXIIIa = Factor XIIIa.

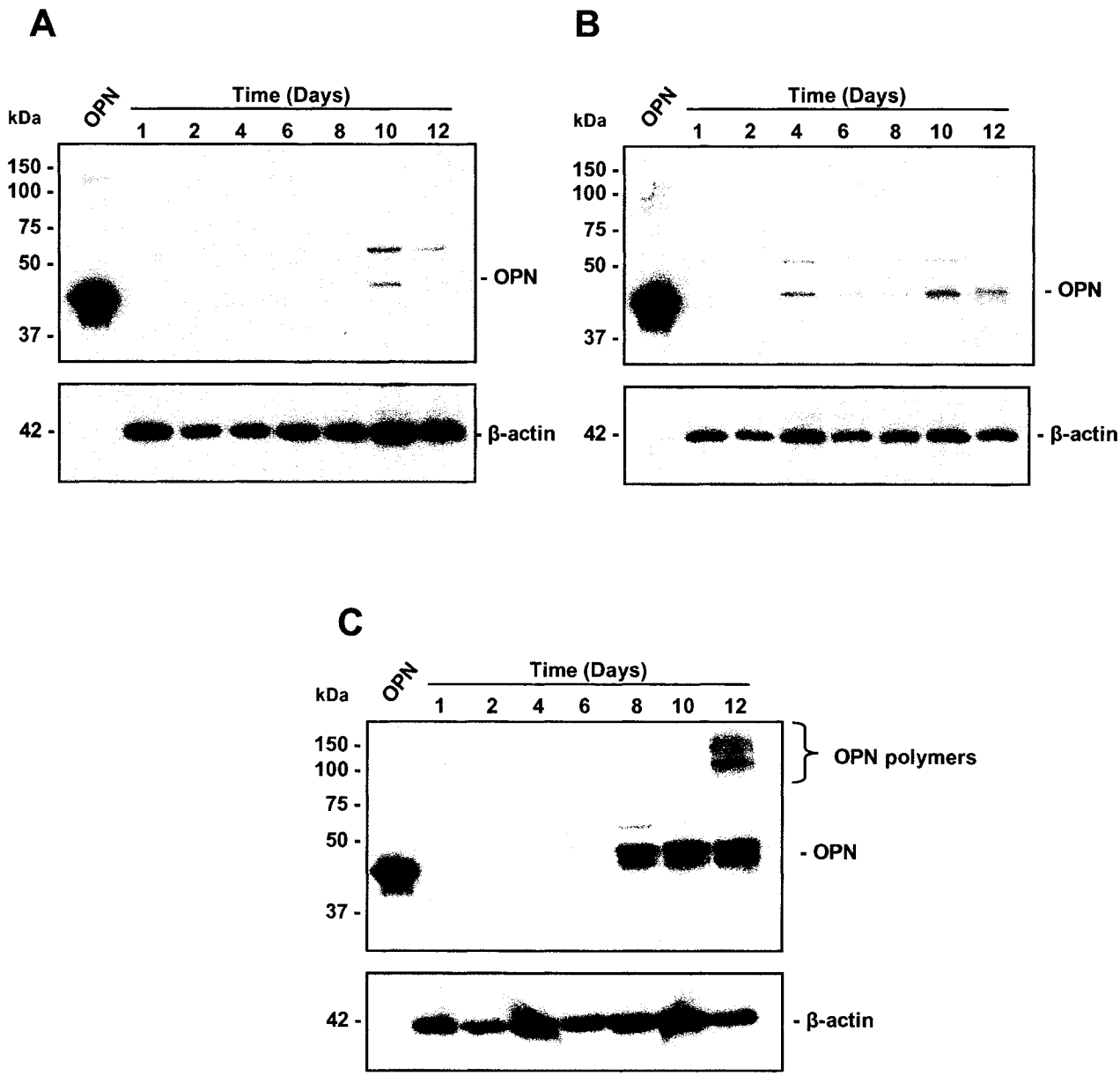



**Figure 24. Osteopontin expression and polymerization in MC3T3-E1/C14 cell extracts assessed by Western blotting.** Cells were cultured in 100 mm dishes and treated with **A)** media only; **B)** AA; or **C)** AA and βGP. At the indicated time points, cells were harvested with Buffer B (with EDTA). 50 μg of total protein was separated by SDS-PAGE and transferred onto PDVF membranes. OPN was detected by immunodetection protocol described in Materials and Methods. The positive control lane (OPN) contains 0.1 μg of rat bone E-extract. β-actin was used as loading control.

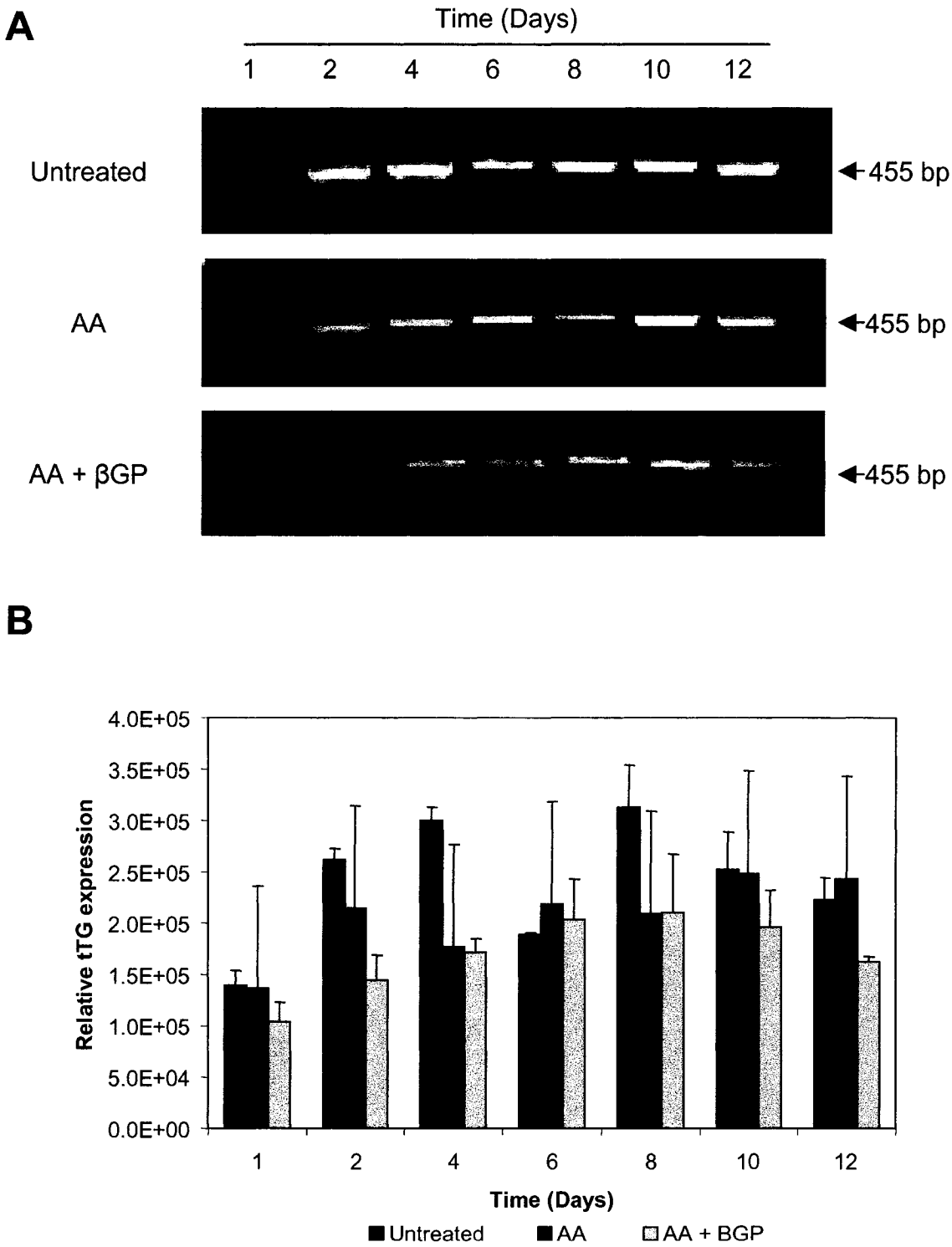



**Figure 25. Tissue transglutaminase (tTG) mRNA expression in MC3T3-E1/C14 cells assessed by RT-PCR. A)** RNA prepared from cells cultured for the indicated periods with, or without, AA and βGP was analyzed by RT-PCR. **B)** Bands were quantified by volume analysis, and GAPDH ratios were used to normalize values.

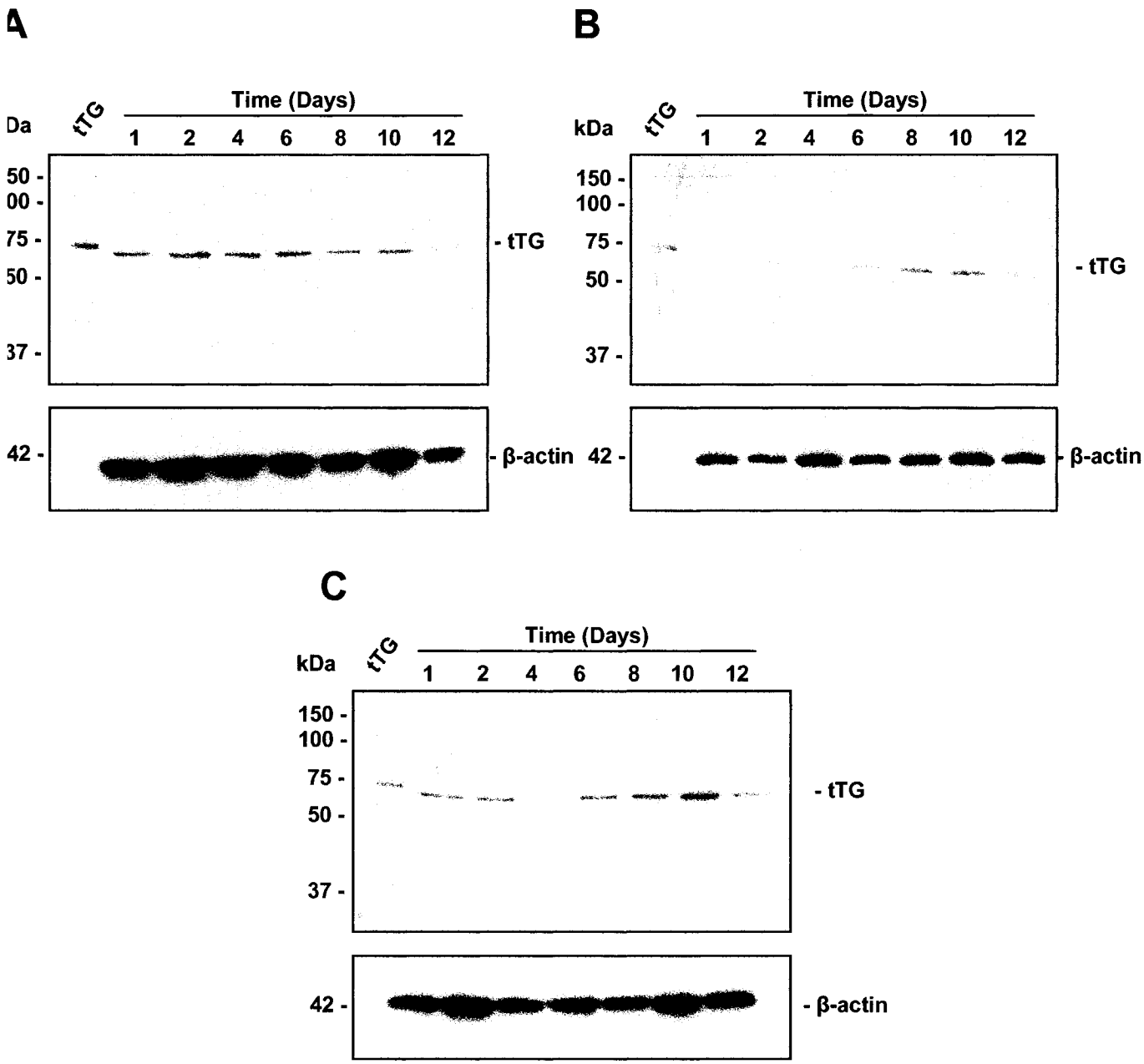



**Figure 26. Tissue transglutaminase expression in MC3T3-E1/C14 cell extracts assessed by Western blotting.** Cells were cultured in 100 mm dishes and treated with **A)** media only; **B)** AA; or **C)** AA and βGP. At the indicated time points, cells were harvested with Buffer A (no EDTA). 50 μg of total protein was separated by SDS-PAGE and transferred onto PDVF membranes. tTG was visualized by immunodetection as described in Materials and Methods. The positive control lane (tTG) contains 5 ng of guinea pig liver tTG standard (Sigma). β-actin was used as a loading control.

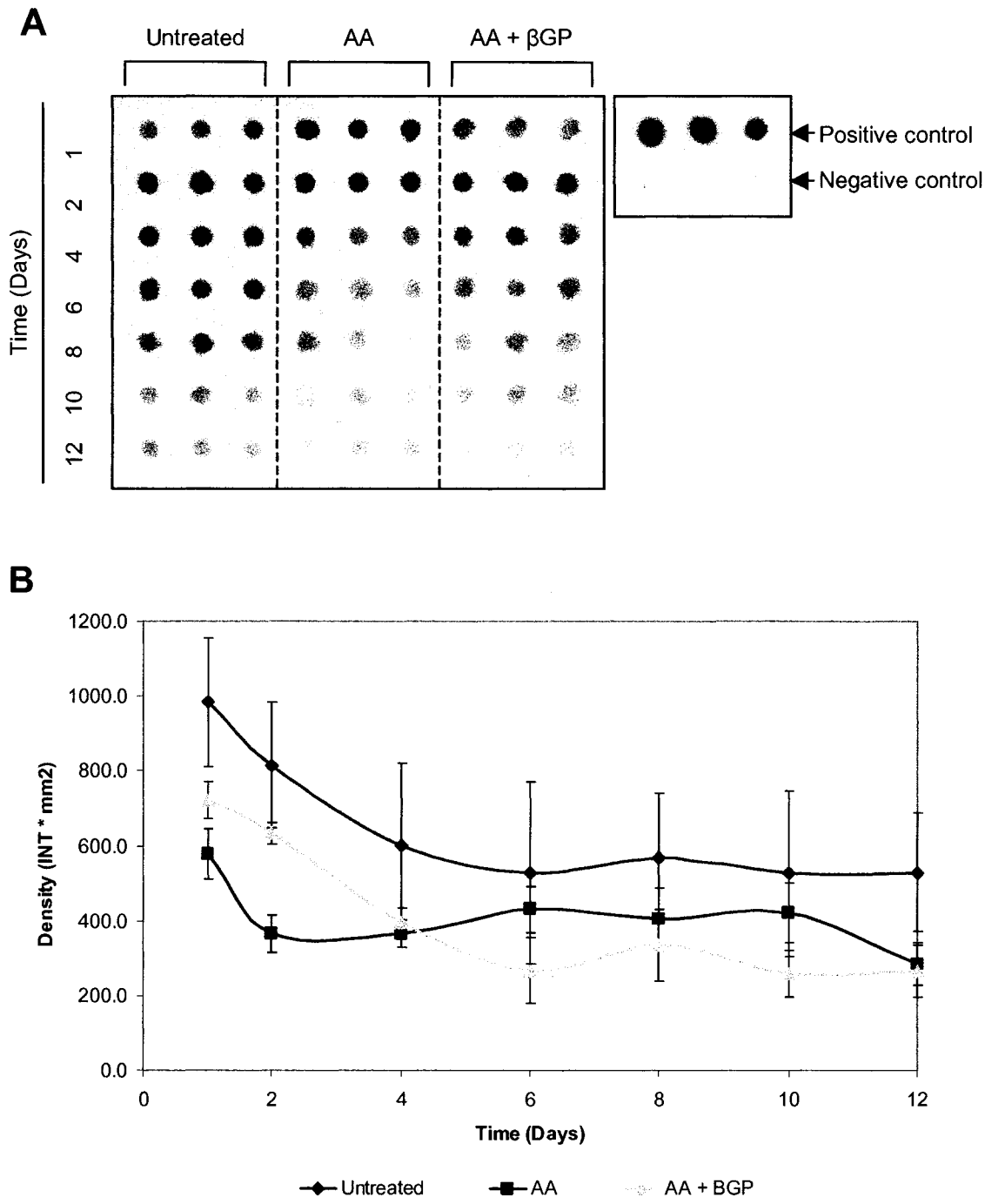



**Figure 27. Tissue transglutaminase activity in MC3T3-E1/C14 cells.** Cells were harvested in Buffer A (no EDTA) and tTG activity was measured by dot blot activity assay using fibronectin as substrate. **A)** Dot blot activity assay showing biotin-pentylamine incorporation into fibronectin, as described in Materials and Methods. **B)** Dots were quantified and analyzed by Quantity OneTM software (Bio-Rad).

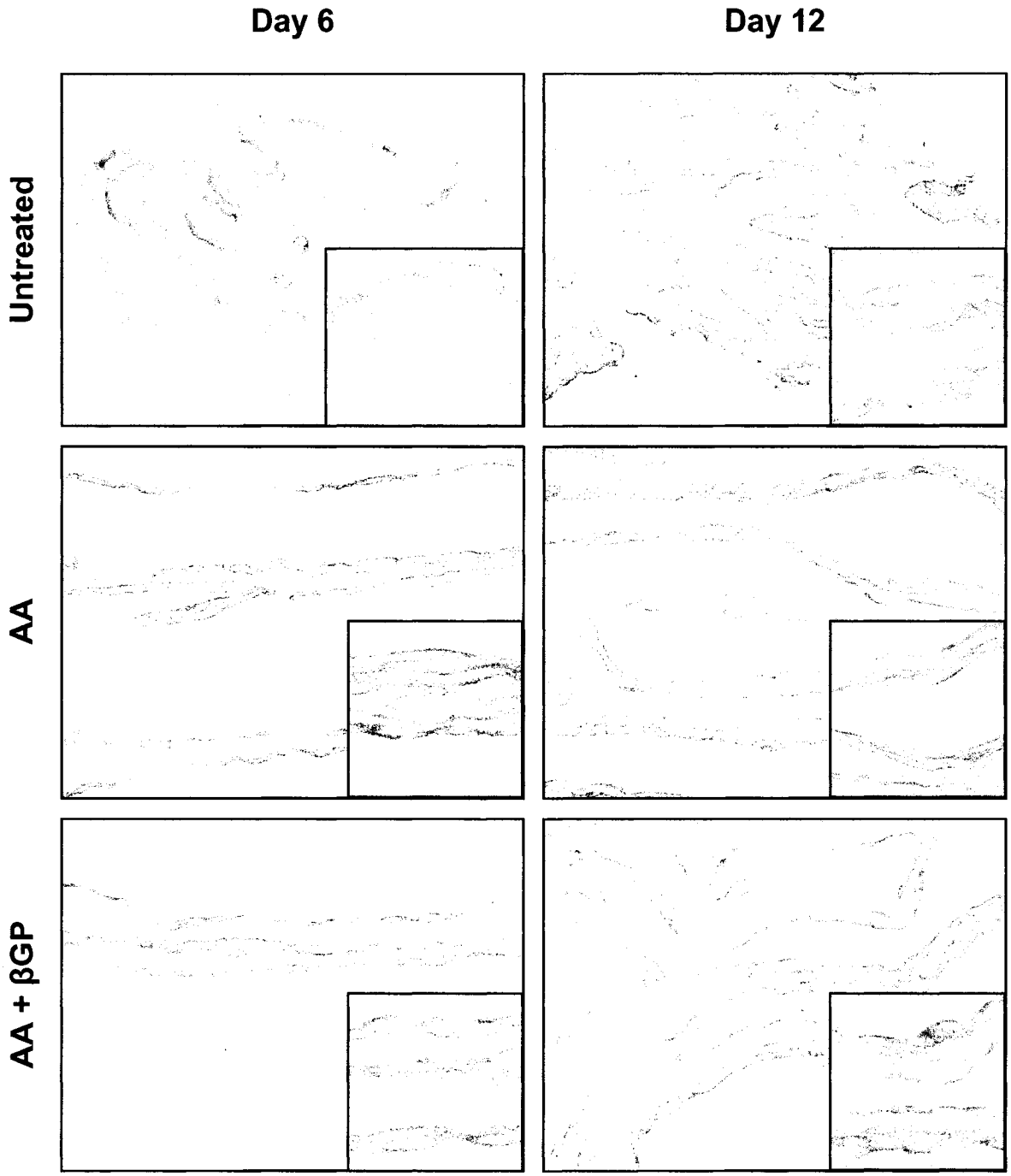



**Figure 28. Immunohistochemical localization of tTG in MC3T3-E1/C14 cells**. Paraffin sections of MC3T3-E1/C14 cells that were grown for 6 and 12 days and treated with medium only, AA, or AA and βGP. Immunostaining for tTG reveals a general staining of the ECM and the cells, independent of experimental conditions tested. Sections were counterstained with methyl green. Negative immunohistochemical staining control is presented in Figure 37. Magnification is x200 (insets x400).

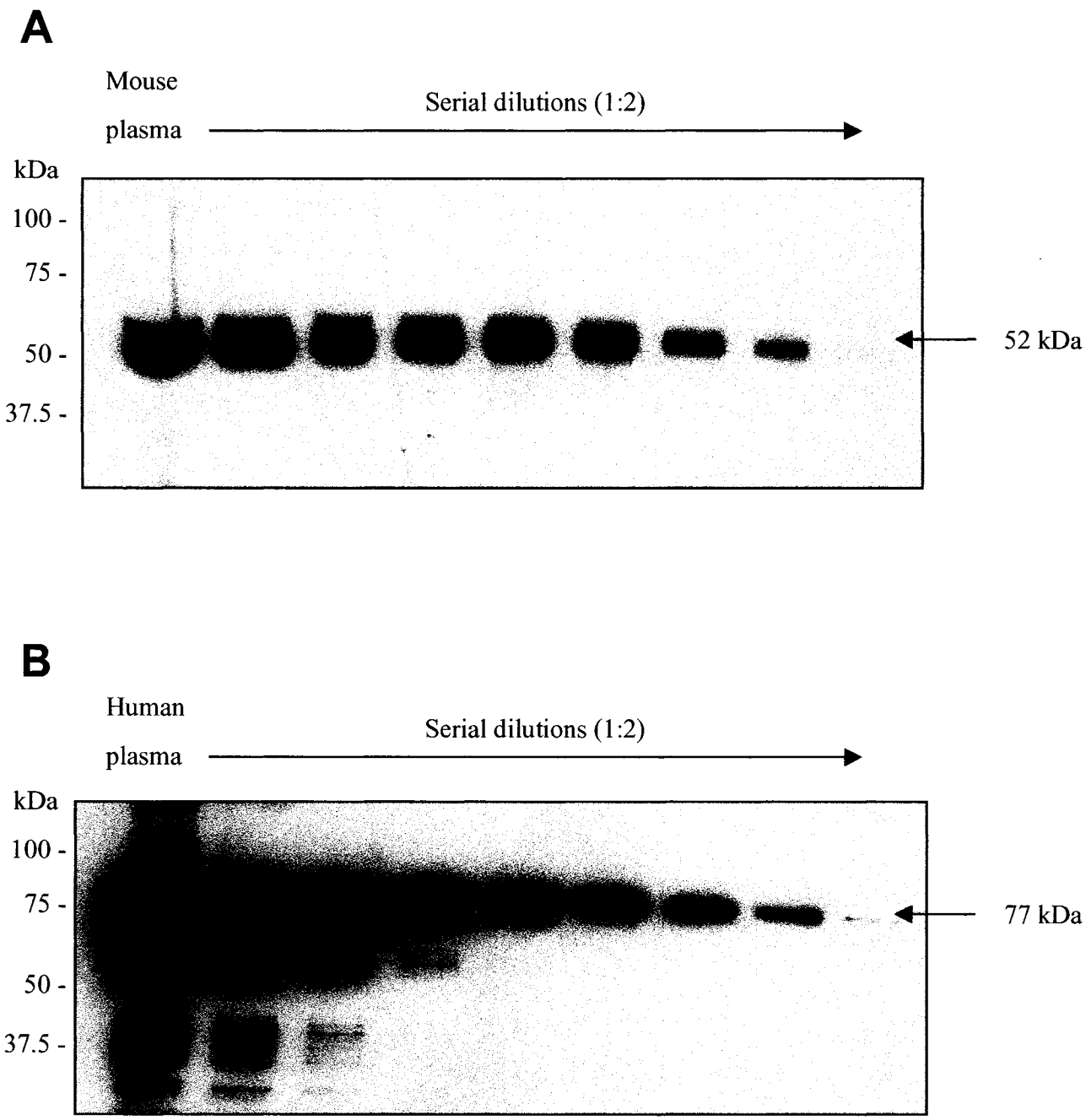



**Figure 29. FXIIIa in mouse and human plasma assessed by Western blotting. A)** mouse plasma serial dilutions; **B)** human plasma serial dilutions. FXIIIa was detected by Western blot using the antibody anti-FXIIIa Ab-1 (AC-1A1) from Neomarkers. The molecular weights of the bands were determined by Quantity One™ software (Bio-RAD) according to the molecular weight marker Precision Plus, also from Bio-RAD.

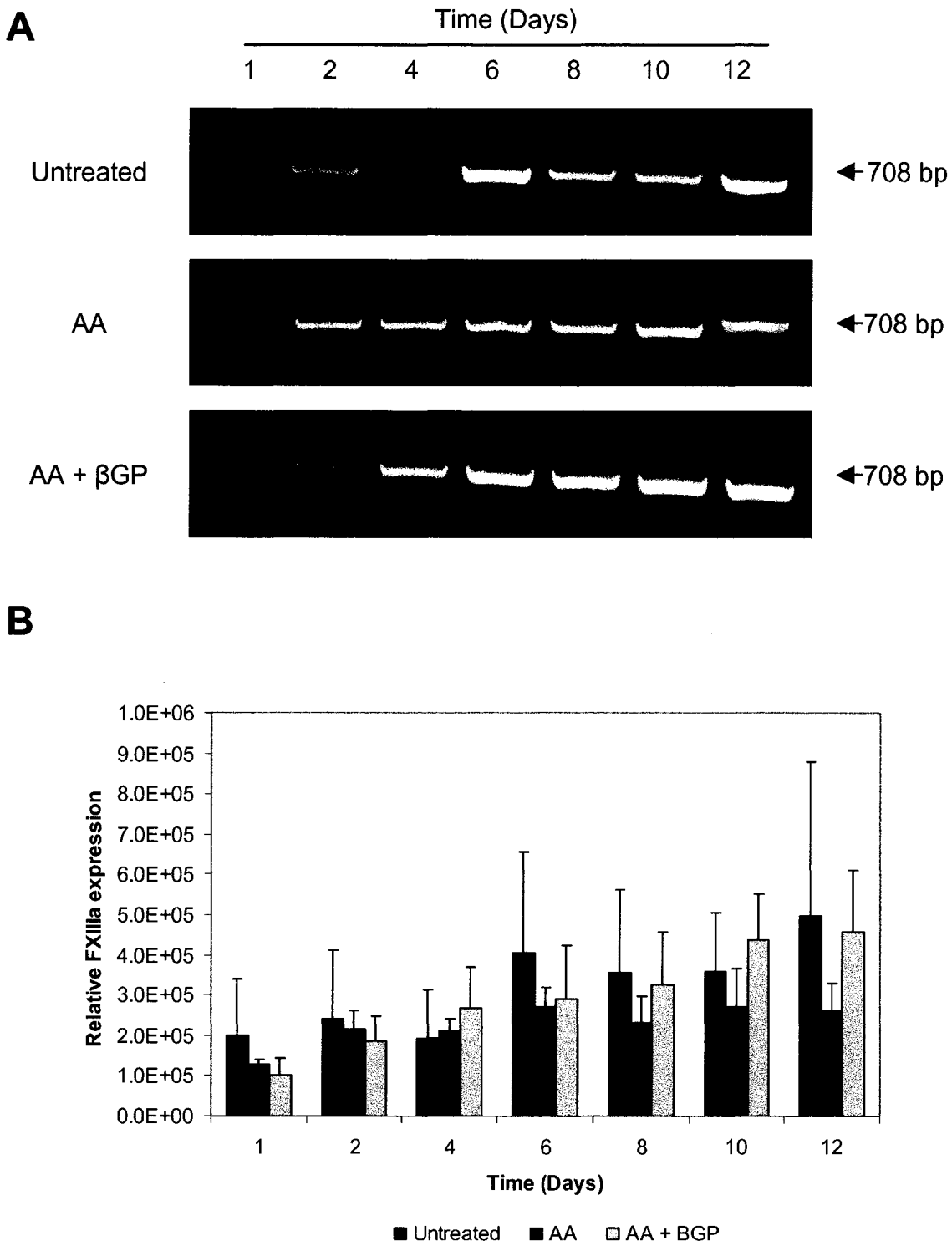



**Figure 30. FXIIIa mRNA expression in MC3T3-E1/C14 cells by RT-PCR. A)** RNA prepared from cells cultured for the indicated periods with, or without, AA and βGP was analyzed by RT-PCR. **B)** Bands were quantified by volume analysis, and GAPDH ratios were used to normalize values.

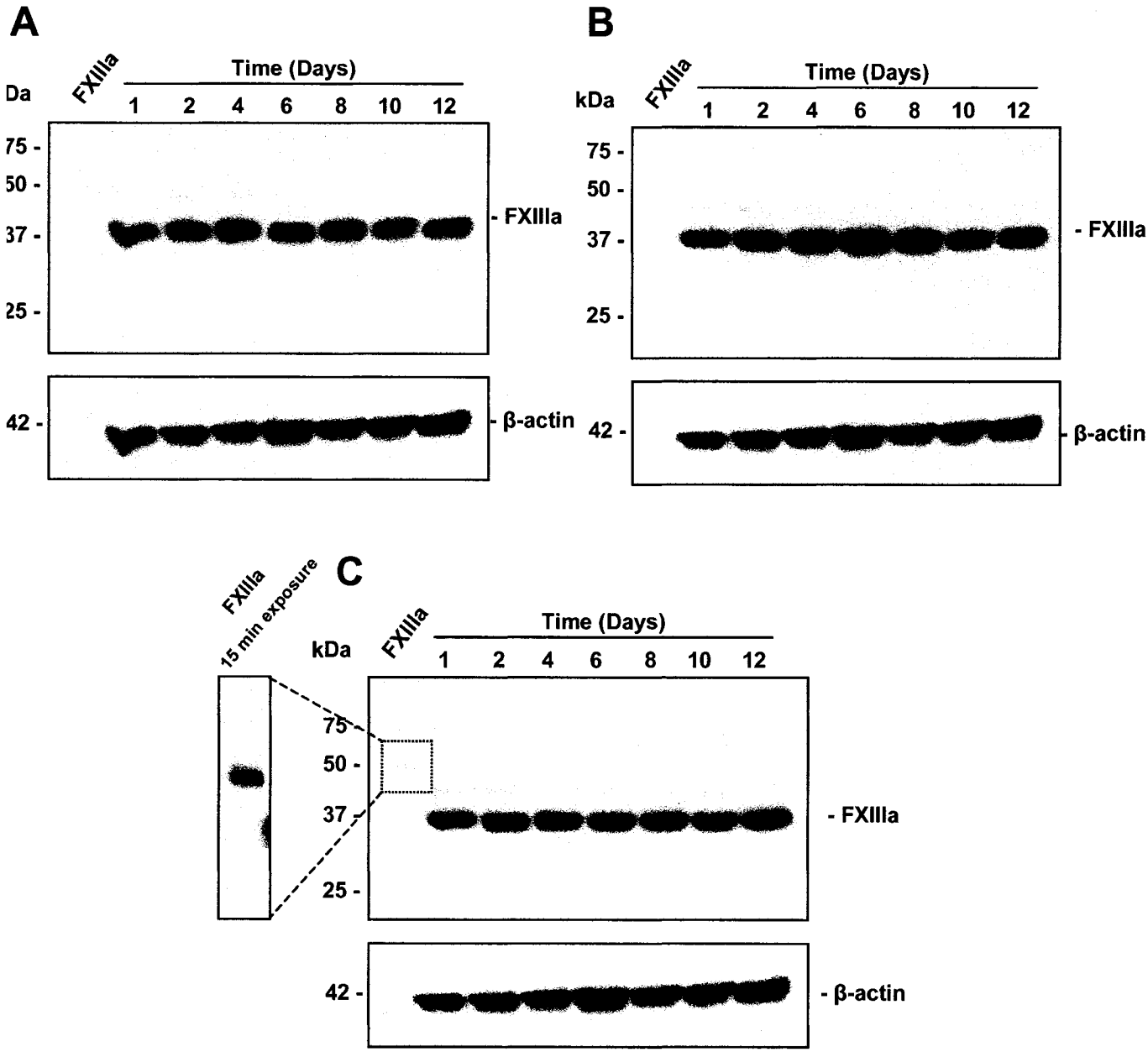



**Figure 31. Western blot of FXIIIa in MC3T3-E1/C14 cells harvested with Buffer A (no EDTA).** Cells were cultured in 100 mm dishes and treated with **A)** media only; **B)** AA or **C)** AA and βGP. At the indicated time points, cells were harvested with Buffer A (no EDTA). 50 μg of total protein was separated by SDS-PAGE and transferred onto PDVF membranes. tTG was detected by immunodetection as described in Materials and Methods. The positive control lane (FXIIIa) contains mouse plasma protein standard. β-actin was used as a loading control.

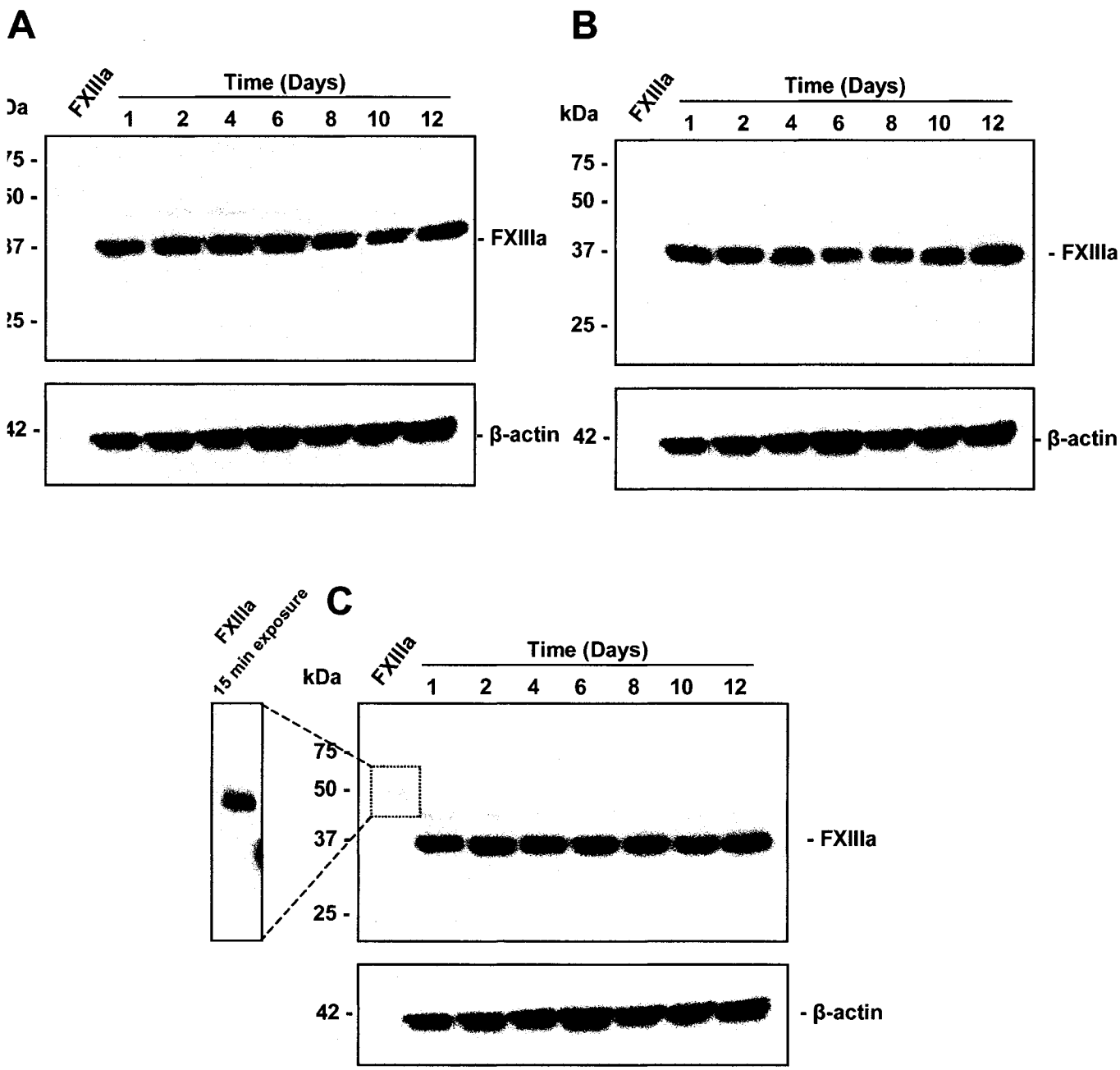



**Figure 32. Western blot of FXIIIa in MC3T3-E1/C14 cells harvested with Buffer B (with EDTA).** Cells were cultured in 100 mm dishes and treated with **A)** media only; **B)** AA or **C)** AA and βGP. At the indicated time points, cells were harvested with Buffer B. 50μg of total protein was separated by SDS-PAGE and transferred onto PDVF membranes. tTG was detected by by immunodetection as described in Materials and Methods. The positive control lane (FXIIIa) contains mouse plasma protein standard. β-actin was used as a loading control.

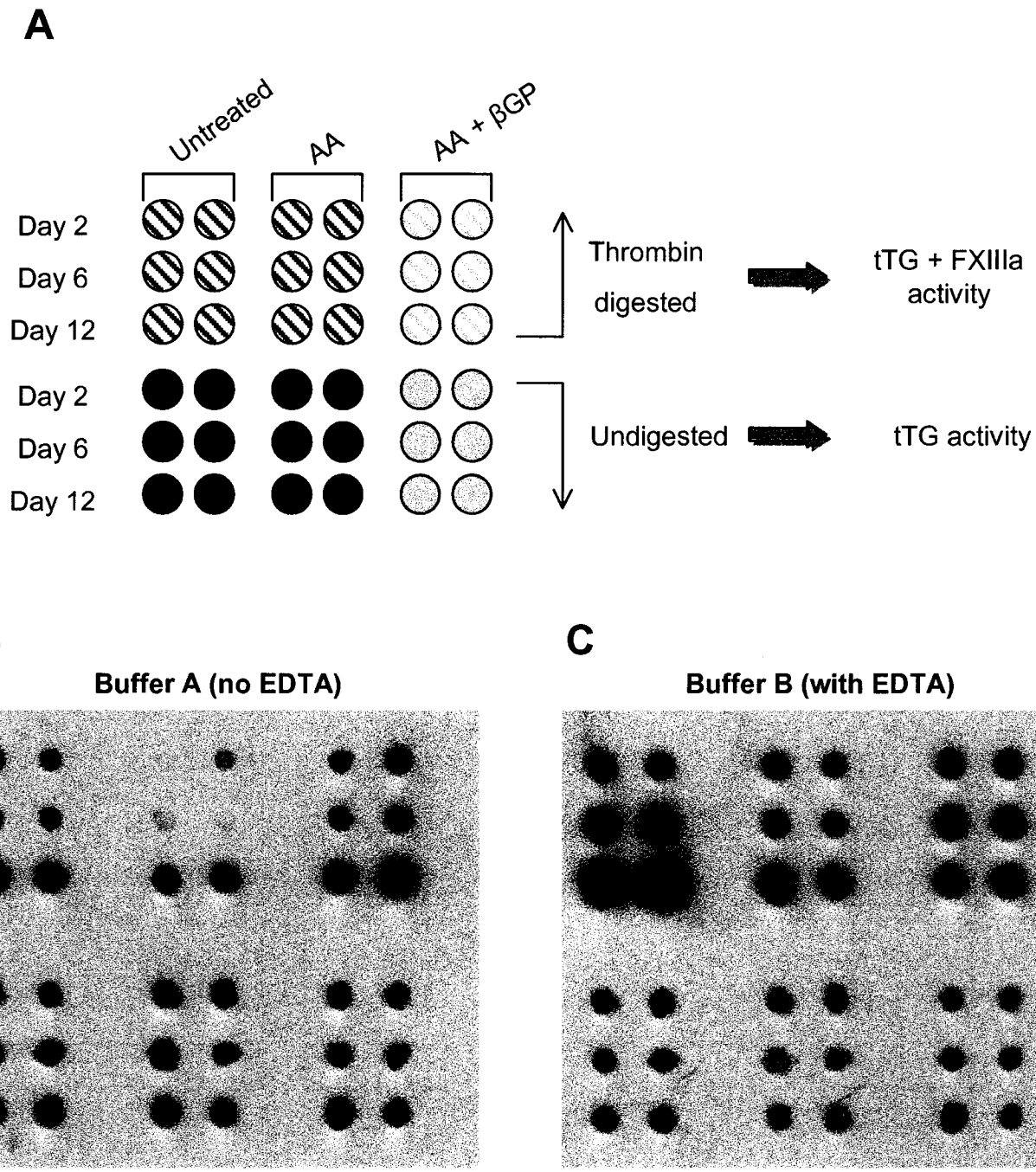



**Figure 33. FXIIIa activity assay. A)** Schema showing the dot blot sample layout. MC3T3-E1/C14 cells were grown in 100 mm dishes for 2, 6, and 12 days and with, or without, AA and βGP. Cells were harvested with **B)** buffer A (no EDTA), or **C)** buffer B (with EDTA). FXIIIa activity was measured by dot blot activity assay. The undigested samples serve as an internal control and indicate the activity attributable to tTG only. Dots were quantified and analyzed by Quantity One™ software (Bio-RAD).

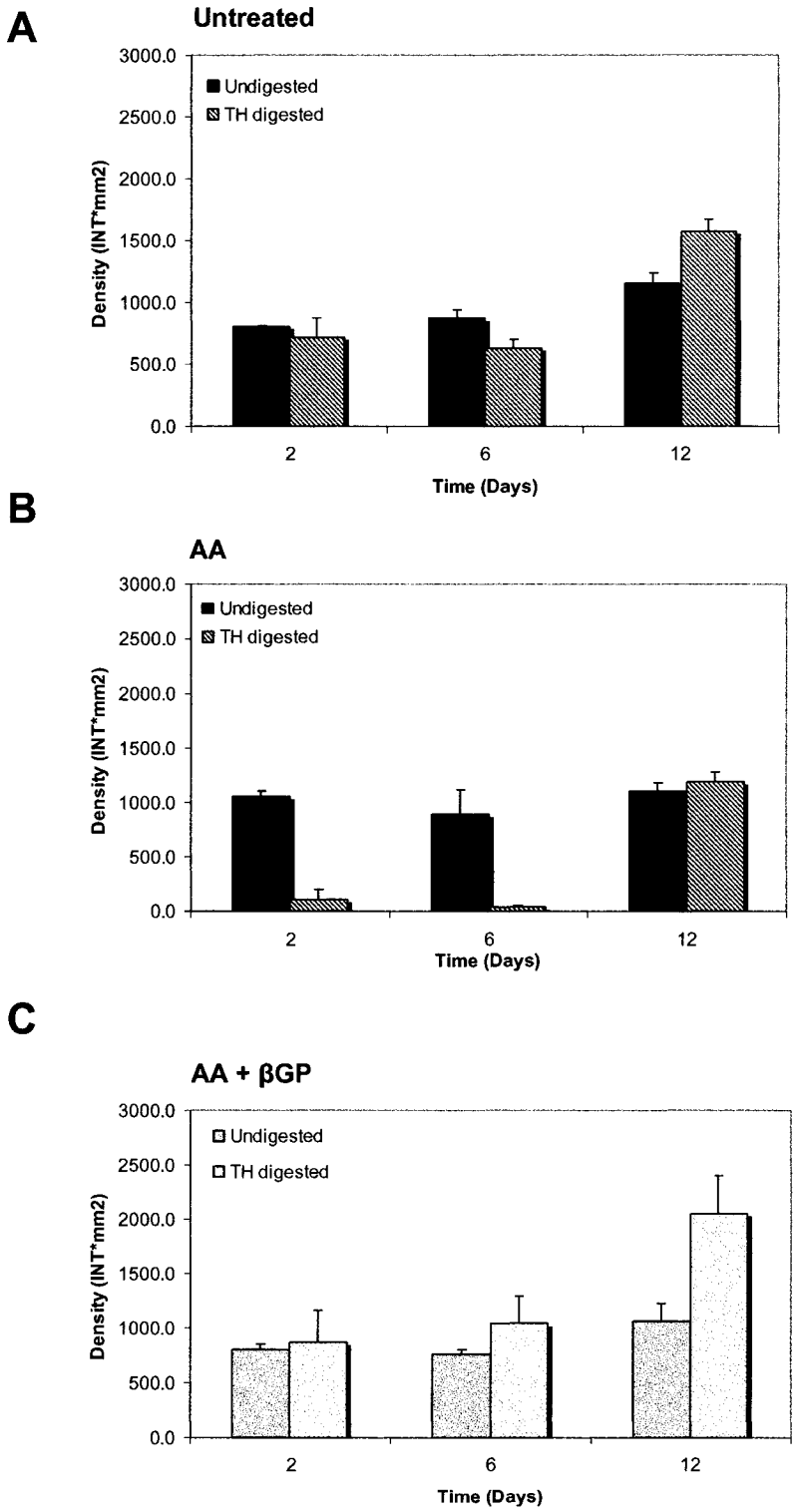



**Figure 34. FXIIIa activity in MC3T3-E1/C14 cells harvested with Buffer A.** MC3T3-E1/C14 cells were grown in 100 mm dishes for 2, 6, and 12 days and treated with **A)** media only; **B)** AA; or **C)** AA and βGP. Cells were harvested with buffer A (no EDTA) and FXIIIa activity was measured by transglutaminase dot blot activity assay.

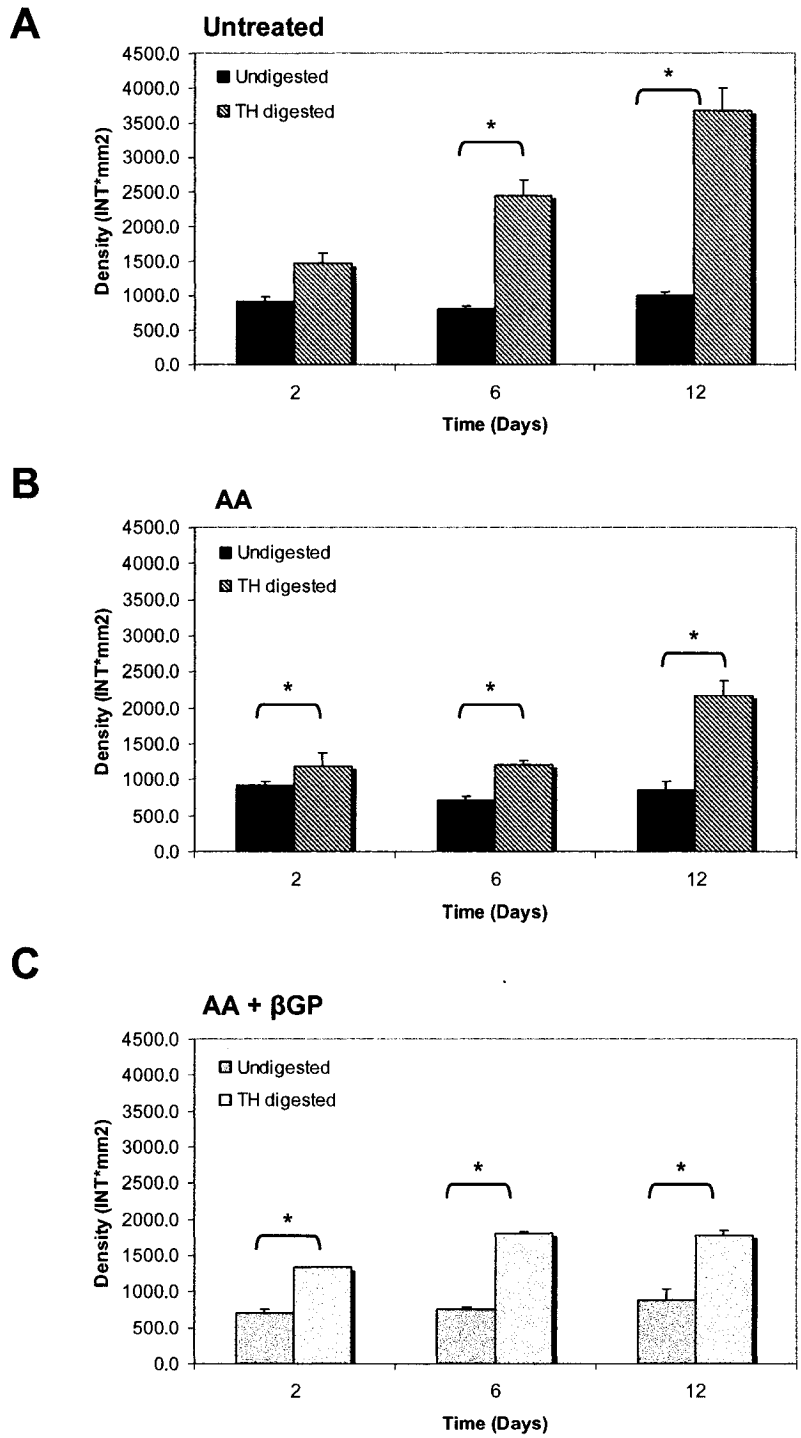
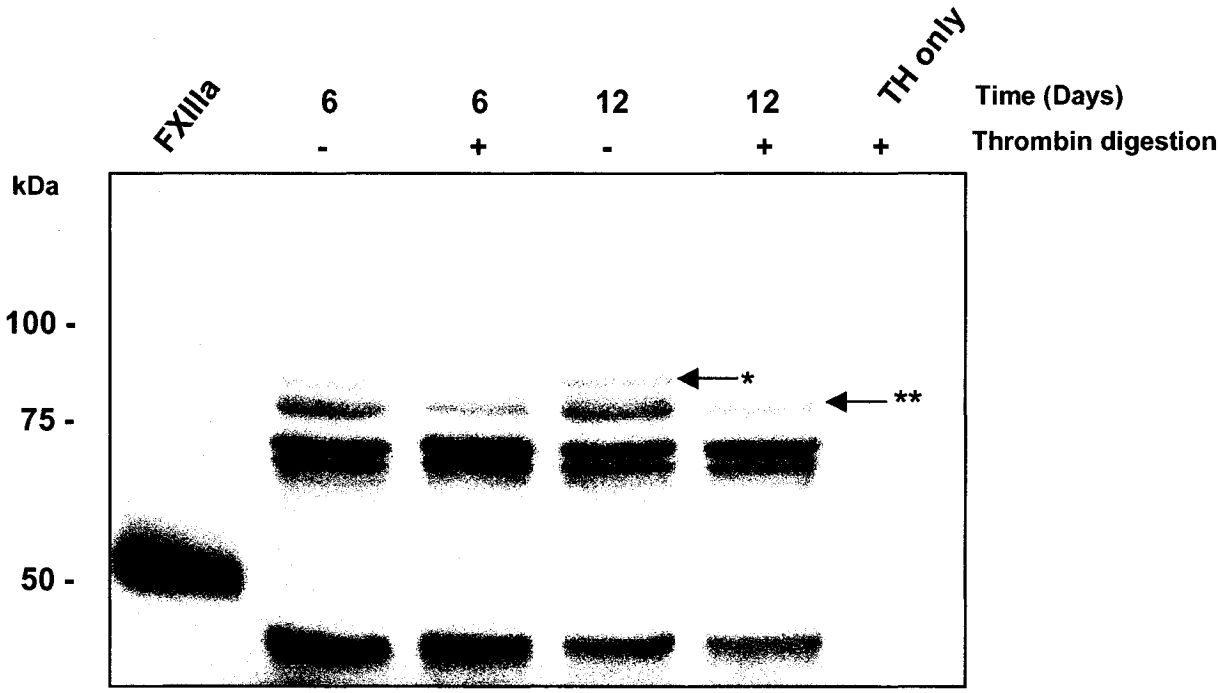



**Figure 35. FXIIIa activity in MC3T3-E1/C14 cells harvested with Buffer B.** MC3T3-E1/C14 cells were grown in 100 mm dishes for 2, 6, and 12 days and treated with **A)** media only; **B)** AA; or **C)** AA and βGP. Cells were harvested with buffer B (with EDTA) and FXIIIa activity was measured by transglutaminase dot blot activity assay. * = $p < 0.1$; $n = 3$.




* = FXIIIa (81kDa)

** = FXIIIa' (77kDa)

**Figure 36. Thrombin cleavage of FXIIIa in MC3T3-E1/C14 cell extracts assessed by Western blotting.** Cells were cultured for 6 and 12 days in complete α-MEM, and harvested with Buffer B (with EDTA). 50 μg of total protein were either pre-digested with thrombin (TH) or left undigested, and then separated by 10% SDS-PAGE, transferred to a PDVF membrane and visualized by immunodetection as described in Materials and Methods. The positive control lane (FXIIIa) contains mouse plasma proteins. The negative control lane (TH only) contains thrombin and reaction buffer (TBS pH 8.0; 3 mM $CaCl_2$). The molecular weights of the bands were determined by Quantity One™ software (Bio-RAD) according to the molecular weight marker Precision Plus, also from Bio-RAD.

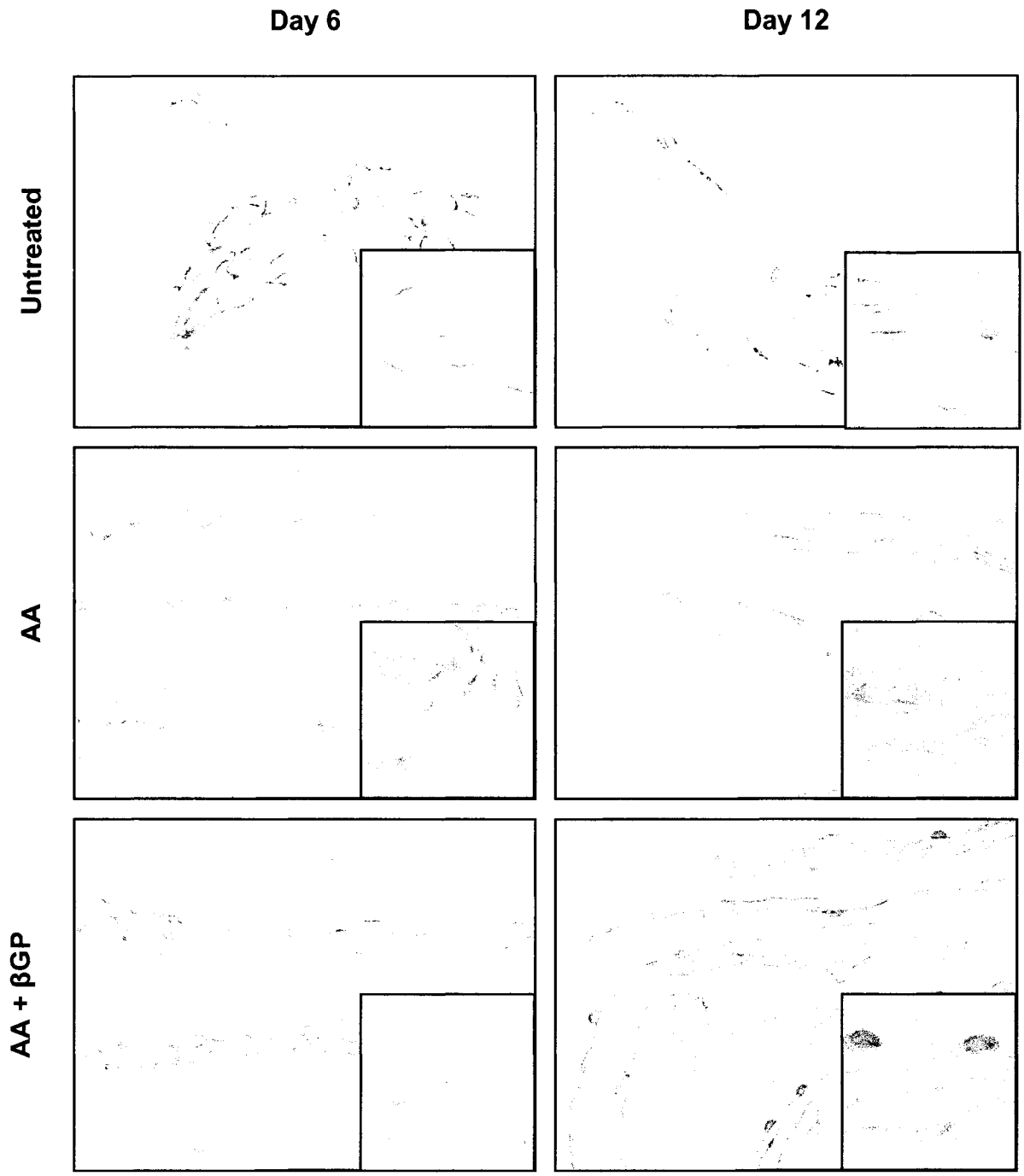



**Figure 37. Immunohistochemical localization of FXIIIa in MC3T3-E1/C14 cells.** Paraffin sections of MC3T3-E1/C14 cells that were grown for 6 and 12 days and treated with media only, AA, or AA and βGP. A strong positive immunostaining (dark pink) for FXIIIa is observed in the cells, whereas the ECM shows a more moderate reaction (pink). Sections were counterstained with methyl green. Negative immunohistochemical staining control is presented in Figure 37. Magnification is x200 (insets x400).

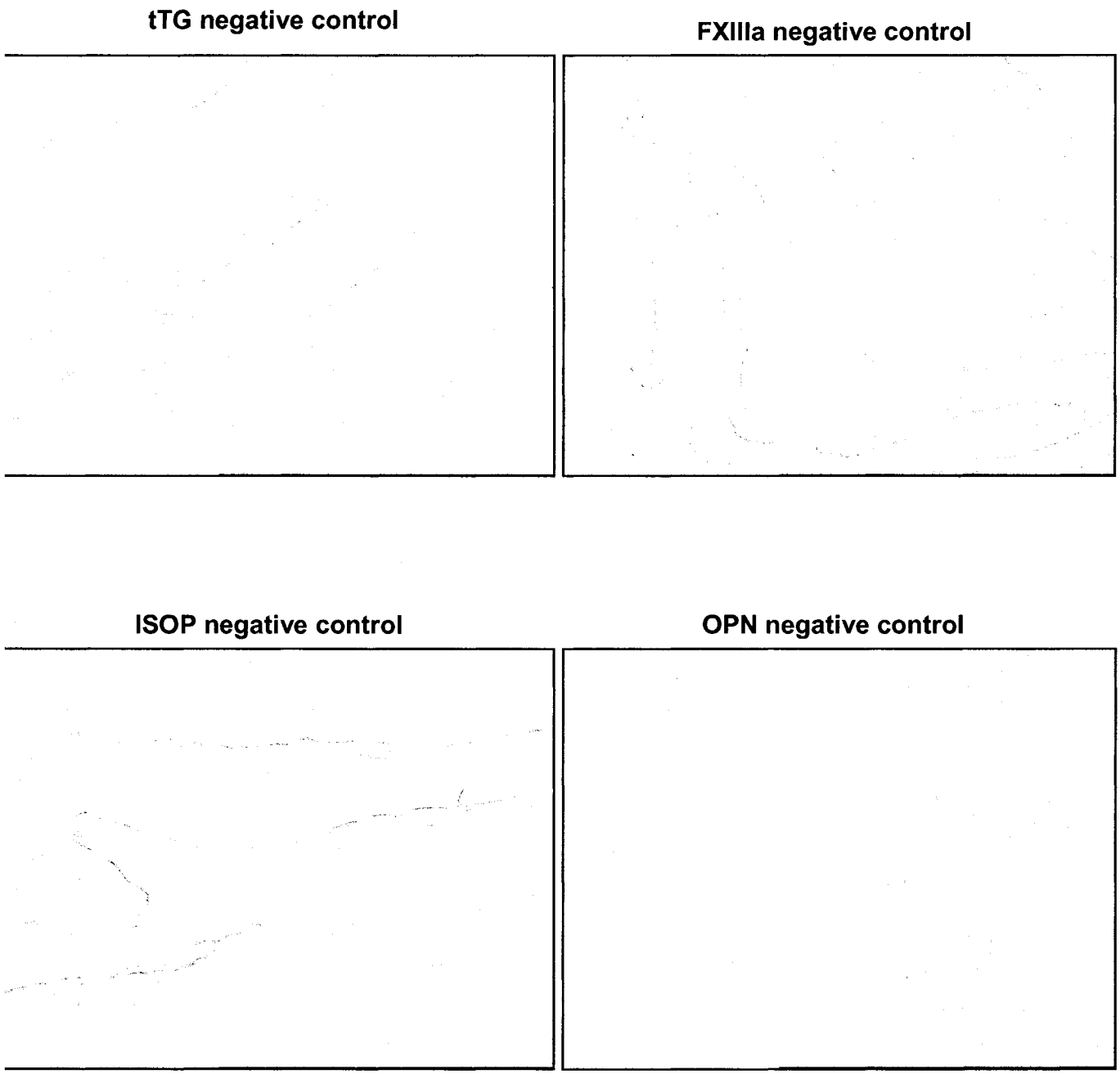



**Figure 38. Immunohistochemical negative controls for tTG, FXIIIa, isopeptide bonds (ISOP), and OPN.** Paraffin sections of MC3T3-E1/C14 cells that were grown for 12 days and treated with AA + βGP. Sections were counterstained with methyl green. Magnification is x200.

# 8. REFERENCES


[1] Landis W.J., **An overview of vertebrate mineralization with emphasis on collagen-mineral interaction.** *Gravit Space Biol Bulletin* (1999) **12**: 15-26.

[2] Boskey A.L. and Paschalis E., **Matrix proteins and biomineralization** in *Bone Engineering*, edited by J.E. Davies, $EM^2$ editions (2000), 655 pages.

[3] Wuttke M., Müller S., Nitsche D.P., Paulsson M, Hanisch F.-G., and Maurer P., **Structural characterization of human recombinant and bone-derived bone sialoprotein.** *J Biol Chem* (2001) **276**: 36839-36848.

[4] Ganss B., Kim R., and Sodek J., **Bone Sialoprotein**. *Crit Rev Oral Biol* (1999) **10**: 78-98.

[5] Stubbs III J.T., Mintz K.P., Eanes E.D., Torchia D.A. and Fisher L.W., **Characterization of native and recombinant bone sialoprotein: delineation of the mineral-binding and cell adhesion domains and structural analysis of the RDG domain.** *J Bone Miner Res* (1997) **12**: 1210-1222.

[6] Hunter G.K. and Goldberg H.A., **Nucleation of hydroxyapatite by bone sialoprotein**. *Proc Natl Acad Sci* (1993) **90**: 8562-8565.

[7] Sodek J., Granss B., and McKee M.D., **Osteopontin**. *Crit Rev Oral Biol Med* (2000) **11**: 279-303.

[8] Butler W.T., Ridall A.L., and McKee M.D., **Osteopontin** *in Principle of bone biology*, edited by Bilezikizn J.P., Raisz L.G. and Rodan G.A., Academic Press, San Diego (1996) pp. 167-181.

[9] McKee M.D. and Nanci A., **Osteopontin: an interfacial extracellular matrix protein in mineralized tissues**. *Connec Tissue Res* (1996) **35**: 251-259.

[10] Lorand L. and Graham R.M., **Transglutaminases: Crosslinking enzymes with pleiotropic functions**. *Nature* (2003) **4**: 140-156.

[11] Liu S., Cerione R.A. and Clardy J., **Structural basis for the guanidine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity.** *PNAS* (2002) **99**: 2743-2747.

[12] Groenen P.J.T.A., Smulders R.H.P.H., Peters R.F.R., Grootjans J.J., Van Den Ijssel P.R.L.A., Bloemendal H and De Jong W.W., **The amine-donor substrate specificity of tissue-type transglutaminase.** *Eur J Biochem* (1994) **220**: 795-799.

[13] Fesus L. and Piacentini M., **Transglutaminase 2: an enigmatic enzyme with diverse functions.** *Trends Biochem Sci* (2002) **27**: 534-539.

[14] Heath D.J., Downes S., Verderio E., and Griffin M., **Characterizetion of tissue transglutaminase in human osteoblast-like cells.** *J Bone Miner Res* (2001) **16**: 1477-1485.

[15] Muesch A., Hartmann E., Rhode K., Rubartelli A., Sitia R. and Rapoport T.A., **A novel pathway for secretory proteins?** *Trends Biochem Sci* (1990) **15**: 86-88.

[16] Chen J.S.K. and Mehta K., **Tissue Transglutamianse: an enzyme with a split personality**. *IJBCB* (1999) **31**: 817-836.

[17] Lu S., Saydak M., Gentile V., Stein J.P. and Davies P.J.A., **Isolation and characterization of the human tissue transglutaminase gene promoter**. *J Biol Chem* (1995) **270**: 9748-9756.

[18] Piacentini M., Ceru M.P., Dini L., Rao M.D., Piredda L., Thomazy V., Davies P.J.A., and Fesus L., **In vivo and in vitro induction of tissue transglutaminase in rat hepatocytes by retinoic acid**. *Biochim Biophys Acta* (1992) **1135**: 171-179.

[19] Aeschlimann D. and Thomazy V., **Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases**. *Connec Tissue Res* (2000) **41**: 1-27.

[20] Hasegawa G., Suwa M., Ichikawa Y., Ohtsuka T., Kumagai S., Kikuchi M., Sato Y and Saito Y., **A novel function of tissue-type transglutaminase: protein disulphide isomerase**. *Biochem J* (2003) **373**: 793-803.

[21] Nanda N., Iismaa S.E., Owens W.A., Husain A., Mackay F. and Graham R.M., **Targeted inactivation of Gh/Tissue Transglutaminase II**. *J Biol Chem* (2001) **276**: 20673-20678.

[22] Griffin M., Casadio R. and Bergamini C.M., **Transglutaminases: Nature's biological glues**. *Biochem J* (2002) **368**: 377-396.

[23] Verderio E., Nicholas B., Gross S., and Griffin M., **Regulated expression of tissue transglutaminase in swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment, and cell death.** *Exp Cell Res* (1998) **239**: 119-138.

[24] Gaudry C.A., Verderio E., Jones R.A., Smith C., and Griffin M., **Tissue transglutaminase is an important player at the surface of human endothelial cells: evidence fir its externalization and its colocalization with the β1 integrin.** *Exp Cell Res* (1999) **252**: 104-113.

[25] Akimov S.S., Krylov D., Fleischmann L.E., and Belkin A.M., **Tisue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin.** *J Cell Biol* (2000) **148**: 825-838.

[26] Akimov S.S. and Belkin A.M., **Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: a role in TGFβ-dependant matrix deposition.** *J Cell Sci* (2001) **114**: 2989-3000.

[27] Prince CW., Dickie D. and Krumdieck C.L., **Osteopontin, a substrate for transglutaminase and FXIII activity.** *Biochem Biophys Res Comm* (1991) **177**: 1205-1210.

[28] Kaartinen M.T., El-Maadawy S., Räsänen N.H. and McKee M.D., **Tissue Transglutaminase and its substrate in bone.** *J Bone Miner Res* (2002) **17**: 2161-2173.

[29] Aeschlimann D., Mosher D. and Paulsson M., **Tissue transglutaminase an factor XIII in cartilage and bone remodeling.** *Semin Thromb Hemostasis* (1996) **22**: 437-443.

[30] Muszbek L, Ádány R. and Mikkola H., **Novel aspects of blood coagulation factor XIII. Structure, distribution, activation and function.** *Crit Rev Clin Lab Sci* (1996) **33**: 357-421.

[31] Marinescu A., Cleary D.B., Littlefield T.R., and Maurer M.C., **Structural features associated with the binding of glutamine-containing peptides to factor XIII.** *Arc Biochem Biophys* (2002) **406**: 9-20.

[32] Lorand L., **FXIII: structure, activation, and interactions with fibrinogen and fibrin.** *Ann N Y Acad Sci* (2000) **936**: 291-311.

[33] Mary A., Achyuthan K.E. and Greenberg C.S., **The binding of divalent metal ions to platelet factor XIII modulates its proteolysis by trypsin and thrombin.** *Arc Biochem Biophys* (1988) **261**: 112-121.

[34] Marinescu A., Cleary D.B., Littlerfield T.R., and Maurer M.C., **Structural features associated with the binding of glutamine-containing peptides to factor XIII.** *Arc Biochem Biophys* (2002) **406**: 9-20.

[35] Lee K.N., Lee C.S., Tae W.-C., Jackson K.W., Christiansen V.J., and McKee P.A., **Crosslinking of $\alpha_2$-antiplasmin to fibrin.** *Annals New York Acad Sci* (2000) 335-339.

[36] Robinson B.R., Houng A.K., and Reed G.L., **Catalytic life of activated factor XIII in thrombi.** *Circulation* (2000) **102**: 1151-1157.

[37] Ádány R. and Bárdos H., **Factor XIII subunit A as an intracellular transglutaminase.** *Cell Mol Life Sci* (2003) **60**: 1049-1060.

[38] Muszbek L., Yee V.C. and Hevessy Z., **Blood coagulation factor XIII: structure and function.** *Thromb Res* (1999) **94**: 271-305.

[39] Nurminskaya M.V and Linsenmayer T.F., **Immunohistological analysis of transglutaminase factor XIIIa expression in mouse embryonic growth plate.** *J Orthop Res* (2002) **20**: 575-578.

[40] Rosenthal A.K., Masuda I., Gohr C.M., Derfus B.A., and Le M., **The transglutaminase, factor XIIIa, is present in articular chondrocytes.** *Osteoarthritis Cartilage* (2001) **9**: 578-581.

[41] Aeschlimann D., Wetterwald A., Fleisch H. and Paulsson M., **Expression of tissue transglutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes.** *J Cell Biol* (1993) **120**: 1461-1470.

[42] Aeschlimann D., Kaupp O. and Paulsson M., **Transglutaminase-catalized matrix crosslinking in differentiating cartilage: identification of osteonectin as a major glutaminyl substrate.** *J Cell Biol* (1995) **129**: 881-892.

[43] Nurminskaya M., Magee C., Nurminsky D. and Linsenmayer T.F., **Plasma transglutaminase in hypertrophic chondrocytes: expression and cell-specific intracellular activation produce cell death and externalization.** *J Cell Biol* (1998) **142**: 1135-1144.

[44] Nurminskaya M.V., Recheis B., Nimpf J., Magee C. and Linsenmayer T.F., **Transglutaminase FXIIIa in the cartilage of developing avian long bones.**, *Dev Dynamics* (2002) **223**: 24-32.

[45] Heath D.J., Downes S., Verderio E., and Griffin M., **Characterizetion of tissue transglutaminase in human osteoblast-like cells.** *J Bone Miner Res* (2001) **16**: 1477-1485.

[46] Nurminskaya M., Magee C., Faverman L., and Linsenmayer T.F., **Chondrocyte-derived transglutaminase promotes maturation of preosteoblasts in periosteal bone.** *Dev Biol* (2003) **263**: 139-152.

[47] Kaartinen M.T., Pirhonen A., Linnala-Kankkunen A., and Mäenpää P.H., **Crosslinking of osteopontin by tissue transglutaminase increases its collagen binding properties.** *J Biol Chem* (1999) **274**: 1729-1735.

[48] Kaartinen M.T., Pirhonen A., Linnala-Kankkunen A., and Mäenpää P.H., **Transglutaminase-catalyzed crosslinking of osteopontin is inhibited by osteocalcin.** *J Biol Chem* (1997) **272**: 22736-22741.

[49] Grigoriadis A.E., Heersede J.N.M. and Aubin J.E., **Differentiation of muscle, fat, cartilage and bone from progenitor cells present in a bone-derived clonal cell population.** *J Cell Biol* (1988)**106**: 2139-2151.

[50] Quarles L.D., Yohay D.A., Lever L.W., Caton R., and Wenstrup R..J., **Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development.** *J Bone Miner Res* (1992) **7**: 683-692.

[51] Aronow M.A., Gerstenfeld L.C., Owen T.A., Tassinari M.S., Stein G.S. and Lian J.B., **Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells.** *J Cell Physiol* (1990) **143**: 213-221.

[52] Kodoma H., Amagai Y., Sudo H., Kasai S. and Yamamoto S., **Establishment of a clonal osteogenic cell line from new-born mouse calvaria.** *Jpn J Oral Biol* (1981) **23**: 899-901.

[53] Wang D., Christensen K., Chawla K., Xiao G., Krebsbach P.H., and Franceschi R.T., **Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential** *J Bone Miner Res* (1999) **14**: 893-903.

[54] Vary C.P.H., Li V., Raouf A., Kitching R., kola I., Franceschi C., Venanzoni M. and Seth A., **Involvement of Ets transcription factors and targets in osteoblast differentiation and matrix mineralization.** *Exp Cell Res* (2000) **257:** 213-222.

[55] Raouf A. and Seth A., **Ets transcription factors and targets in osteogenesis.** *Oncogene* (2000) **19**: 6455-6463.

[56] Franceschi R.T. and Iyer B.S., **Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells.** *J Bone Miner Res* (1992) **7**: 235-246.

[57] Belows C.G., Aubin J.E., ,and Heersche J.N.M., **Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro.** *Endocrinology* (1987) **121**: 1985-1992.

[58] Otsuka E., Yamaguchi A., Hirose S. and Hagiwara H., **Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid.** *Am J Physiol* (1999) **277**: 132-138.

[59] Bortell R., Owen T.A., Shalhoub V., Henrichs A., Aronow M.A., Rochette-Egly C., Lutz Y., Stein J.L., Lian J.B. and Stein G.S., **Constitutive transcription of the osteocalcin gene in osteosarcoma cells is reflected by altered protein-DNA interactions at promoter regulatory elements.** *Proc Natl Acad Sci* (1993) **90**: 2300-2304.

[60] Huang W., Carlsen B., Rudkin G.H., Shah N., Chung C., Ishida K., Yamaguchi D.T. and Miller T.A., **Effect of serial passage on gene expression in MC3T3-E1 preosteoblastic cells: a microarray study.** *Biochem Biophys Res Comm* (2001) **281**: 1120-1126.

[61] Grigoriadis A., PMP. Ber R., Aubin J., and Weersche J., **Subclone heterogeneity in a clonally derived osteoblast-like cell line.** *Bone* (1985) **6**: 249-256.

[62] Xiao G., Cui Y., Ducy P., Karsenty G., and Franceschi R.T., **Ascorbic acid-dependant activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence.** *Mol Endocrinol* (1997) **11**: 1103-1113.

[63] Leis H., Hulla W., Gruber R., Huber E., Zach D., Gleispach H., and Windischhofer W., **Phenotypic heterogeneity of osteoblast-like MC3T3-E1 cells: changes in braykinin-induced prostaglandin $E_2$ production during osteoblast maturation.** *J Bone Miner Res* (1997) **12**: 541-551.

[64] Tullberg-Reinert H. and Jundt G., **In situ measurement of collagen synthesis by human bone cells with a Sirius red-based colorimetric microassay: effects of transforming growth factor β2 and ascorbic 2-phosphate.** *Histochem Cell Biol* (1999) **112**: 271-276.

[65] Heinonen J.K. and Lahti R.J., **A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphate.** *Anal Biochem* (1981) **113**: 313-319.

[66] Laird P.W., Zijderveld A., Linders K., Rudnicki M.A., Jaenisch R. and Berns A., **Simplified mammalian DNA isolation procedure.** *Nucleic Acid Res* (1991) **19**: 4293.

[67] Schneider W.C., **Determination of nucleic acids in tissues by pentose analysis.** *Methods Enzymol* (1957) **3**: 680-684.

[68] Chung C.Y., Iida-Klein A., Wyatt L.E., Rudkin G.H., Ishida K., Yamaguchi D.T., and Miller T.A., **Serial passage of MC3T3-E1 cells alters osteoblastic function and responsiveness to transforming growth factor-β1 and bone morphogenic protein-2.** *Biochem Biophys Res Com* (1999) **265**: 246-251.

[69] Saito Y., Yoshizawa T., Takizawa F., Ikegame M., Ishibashi O., Okuda K., Hara K., Ishibashi K., Obinata M., and Kawashima H., **A cell line with characteristics of the periodontal ligament fibroblasts is negatively regulated for mineralization and Runx2/Cbfa1/Osf2 activity, part of which can be overcome by bone morphogenic protein-2.** *J Cell Sci* (2002) **115**: 4191-4200.

[70] Ehara A., Ogata K., Imazato S., Ebisu S., Nakano T., and Umakoshi Y., **Effects of α-TCP and TetCP on MC3T3-E1 proliferation, differentiation and mineralization.** *Biomaterials* (2003) **24**: 831-836.

[71] Nonaka M., Matsuda Y., Shiroishi T., Moriwaki K., Nonaka M., and Natsuume-Sakai S., **Molecular cloning of the b subunit of mouse coagulation factor XIII and assignment of the gene to chromosome 1: close evolutionary relationship to complement factor H.** *Genomics* (1993) **15**: 535-542.

[72] Citron B.A., Gregory E.J., Steigerwalt D.S., Qin F., and Festoff B.W., **Regulation of the dual function tissue transglutaminase/$G\alpha_h$ during murine neuromuscular development: gene and enzyme isoform expression.** *Neurochem Int* (2000) **37**: 337-349.

[73] Hitomi K., Horio Y., Ikura K., Yamanishi K., and Maki M., **Analysis of epidermal-type transglutaminase (TGase 3) expression in mouse tissues and cell lines.** *Int J Biochem Cell Biol* (2001) **33**: 491-498.

[74] Candi E., Oddi S., Terronini A., Paradisi A., Ranalli M., Finazzi-Agró A., and Melino G., **Transglutaminase 5 cross-links loricrin, involucrin, and small proline-rich proteins *in Vitro*.** *J Biol Chem* (2001) **276**: 35014-35023.

[75] Aubin J.E. and Liu F., **The osteoblast lineage** *in Principle of bone biology*, edited by Bilezikizn J.P., Raisz L.G. and Rodan G.A., Academic Press, San Diego (1996) pp. 51-67.

[76] Whyte M.P., **Hypophosphatasia: Nature's window on alkaline phosphatase function in man** *in Principle of bone biology*, edited by Bilezikizn J.P., Raisz L.G. and Rodan G.A., Academic Press, San Diego (1996) pp. 951-968.

[77] Hui M. and Tenenbaum H.C., **New face of an old enzyme: alkaline phosphatase may contribute to human tissue aging by inducing tissue hardening and calcification.** *New Anat* (1998) 91-94.

[78] Aubin J.E., **Advances in the osteoblast lineage.** *Biochem cell biol* (1998) **76**: 899-910.

[79] McKee M.D. and Nanci A., **Osteopontin: an interfacial extracellular matrix protein in mineralizing tissues.** *Connect Tissue Res* (1996) **35**: 197-205

[80] Lai T.-S., Slaughter T.F., Peoples K.A., and Greenberg C.S., **Site-directed mutagenesis of the calcium-binding site of blood coagulation factor XIIIa.** *J Biol Chem* (1999) **274**: 24953-24958.

[81] Lorand L., **Factor XIII: structure, activation, and interactions with fibrinogen and fibrin.** *Annals NY Acad Sci* (2000) **936**: 291-311.

[82] Ronchetti, I.P., Quaglino D.Jr. and Bergamini G., **Ascorbic acid and connective tissue** in *Subcellular biochemistry, Volume 25: Ascorbic acid: Biochemistry and Biomedical cell biology*, edited by Harris J.R., Plenum Press, New York (1996) pp 1-10.

[83] Yu K., Kurata T. and Arakawa N., **The behavior of L-ascorbic acid in the prolyl 4-hydroxalase reaction**. *Agric Biol Chem* (1988) **52**: 729-731.

[84] Xiao. G., Gopalakrishnan R., Djiang D., Reith E., Benson M.D. and Franceschi R.T., **Bone morphogenic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells**. *J Bone Miner Res* (2002) **17**: 101-110.

[85] Candeliere G.A., Liu F. and Aubin J.E., **Heterogeneity of marker expression by osteoblasts in different zones of bone in the 21 day fetal rat calvaria**. *J Bone Miner Res* (1997) **12**: S187.

[86] Verderio E., Gaudry C., Gross S., Smith C., Downes S. and Griffin M., **Regulation of cell surface tissue transglutaminase: effects on matrix storage of latent transforming growth factor-β binding protein-1.** *J Histochem Cytochem* (1999) **47**: 1417-1432.

[87] Gentile V., Thomazy V., Piacentini M., Fesus L. and Davies P.J.A., **Expression of tissue transglutaminase in Balb-C 3T3 fibroblasts: effects on cellular morphology and adhesion**. *J Cell Biol* (1992) **119**: 463-474.

[88] Gaudry .C.A., Verderio E., Jones R.A., Smith C. and Griffin M., **Tissue transglutaminase is an important player at the surface of human endothelial cells: evidence for its externilization and its colocalization with the β1 integrin.** *Exp Cell Res* (1999) **252**: 104-113.

[89] Hiiragi T., Sasaki H., Nagafuchi A., Sabe H., Shen S.C., Matsuki M., Yamanishi K., and Tsukita S, **Transglutaminase type 1 and its crosslinking activity are concentrated at adherens junctions in simple epithelial cells.** *J Biol Chem* (1999) **274**: 34148-34154.

[90] Ueki S., Takagi J. and Saito Y., **Dual functions of transglutaminase in novel cell adhesion**. *J Cell Sci* (1996) **109**: 2727-2735.

[91] Paye M. and Lapiere C.M., **The lack of attachment of transformed embryonic lung epithelial cells to collagen I is corrected by fibronectin and FXIII.** *J Cell Sci* (1986) **86**: 95-107.

[92] Paye M., Nusgens B.V. and Lapiere C.M., **Factor XIII of blood coagulation modulates collagen biosynthesis by fibroblasts in vitro.** *Haemostasis* (1989) **19**: 274-283.

[93] De Laurenzi V. and Melino G., **Gene disruption of tissue transglutaminase**. *Mol Cell Biol* (2001) **21**: 148-155.

[94] Lauer P., Metzner H.J., Zettmeibl G., Li M., Smith A.G., Lathe R., and Dickneite G., **Targeted inactivation of the mouse locus encoding coagulation factor XIII-A: hemostatic abnomalities in mutant mice and characterization of the coagulation deficit.** *Thromb Haemost* (2002) **88**: 967-974.

[95] Grynpas M.D., **Fluoride effects on bone crystals**. *J Bone Miner Res* (1990) **5 (S1)**: S169-S175.